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HYDROLYSIS REACTION OF NUTRIENTS FROM MICROALGAE

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สุดารัตน์ ภู่กลาง : การสกัดสารอาหารจากจุลสาหร่ายด้วยปฏิกิริยาไฮโดรไลซิส. (HYDROLYSIS REACTION OF NUTRIENTS FROM MICROALGAE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.ประเสริฐ ภวสันต์, 56 หน้า.

งานวิจัยนี้ทำการทดลองเกี่ยวกับปฏิกิริยาไฮโดรเทอร์มอลไฮโดรไลซิสคาร์โบไฮเดรตของสาหร่าย *Scenedesmus* sp. และ *Ankistrodesmus* sp. ทั้งก่อนและหลังกระบวนการสกัดไขมัน คาร์โบไฮเดรตเหล่านี้จะถูกไฮโดรไลซ์ที่สภาวะน้ำกึ่งวิกฤติเป็นน้ำตาลรีดิวส์ภายในเครื่องปฏิกรณ์แบบกะชนิดหม้ออัดความดัน ในส่วนแรกทำการทดลองหาสภาวะที่เหมาะสมของไฮโดรไลซิสสาหร่ายก่อนนำไปสกัดไขมัน ได้แก่ ความเข้มข้นของชีวมวล (10-40 กรัม/ลิตร), อุณหภูมิ (180–220 องศาเซลเซียส), เวลา (0.5-2 ชั่วโมง), ชนิดของกรด (กรดไฮโดรคลอริก, กรดไนตริก และกรดซัลฟิวริก) และความเข้มข้นกรด (0-2% โดยน้ำหนัก) ปริมาณกลูโคสสูงสุดที่ได้จาก *Scenedesmus* sp. และ *Ankistrodesmus* sp. เกิดที่ความเข้มข้นกรดซัลฟิวริก 0.75% โดยน้ำหนักที่อุณหภูมิ 180 องศาเซลเซียสโดยใช้เวลา 180 นาทีคือ 5.96 และ 2.38 กรัม/ลิตร ตามลำดับ เมื่อเพิ่มความเข้มข้นกรดให้สูงขึ้นปริมาณกรดน้ำตาลที่เกิดจากการย่อยของน้ำตาล เช่น กรดแอซิติก, เฟอฟูรอล, ไฮดรอกซีเมทิลเฟอฟูราน, กรดเลวูลินิค และกรดฟอร์มิกมีปริมาณเพิ่มมากขึ้น ปริมาณไขมันที่ได้จากกากสาหร่าย *Scenedesmus* sp. และ *Ankistrodesmus* sp. ที่ผ่านการไฮโดรไลซิสที่สภาวะนี้ คือ 13.78 และ 24.88% โดยน้ำหนัก

ส่วนที่สองเกี่ยวกับการไฮโดรไลซิสของกากสาหร่ายที่ได้ภายหลังการสกัดไขมัน ไขมันที่ได้จากการสกัด *Scenedesmus* sp. คือ 14.9% โดยน้ำหนัก และจาก *Ankistrodesmus* sp. คือ 28.14% โดยน้ำหนัก การไฮโดรไลซิสกากสาหร่ายที่ได้ภายหลังการสกัดไขมัน พบว่าจะให้กลูโคสสูงสุดที่สภาวะความเข้มข้นกรดซัลฟิวริก 0.5% โดยน้ำหนักที่อุณหภูมิ 200 องศาเซลเซียสและใช้เวลา 120 นาที เท่ากับ 6.08 กรัม/ลิตร (*Scenedesmus* sp.) และ 1.89 กรัม/ลิตร (*Ankistrodesmus* sp.) ปริมาณกรดน้ำตาลที่ได้จากสภาวะนี้พบว่าคล้ายคลึงกับที่ได้จากการทดลองแบบไฮโดรไลซิสก่อนการสกัดไขมันยกเว้นในขั้นตอนนี้กรดฟอร์มิกจะไม่เกิดขึ้น เมื่อเปรียบเทียบกันพบว่าการไฮโดรไลซิสกากที่ได้จากการสกัดไขมันจะช่วยลดปริมาณการใช้กรดและเวลาที่ใช้ในขั้นตอนการไฮโดรไลซิสได้เพราะสาหร่ายได้รับการปรับสภาพจากตัวทำละลายและความร้อน จากการทดลองทั้งหมดทำให้ทราบว่าปริมาณน้ำตาลและกรดน้ำตาลที่ได้ขึ้นกับสภาวะที่แตกต่างกันของการไฮโดรไลซิส

ภาควิชา วิศวกรรมเคมี ลายมือชื่อนิสิต .....

สาขาวิชา วิศวกรรมเคมี ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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SUDARAT PHUKLANG: HYDROLYSIS REACTION OF NUTRIENTS FROM MICROALGAE. ADVISOR: ASSOC. PROF. PRASERT PAVASANT, Ph.D., 56 pp.

This work investigated the efficiency of the hydrothermal hydrolysis reaction of *Scenedesmus* sp. and *Ankistrodesmus* sp. carbohydrate before/after lipid extraction. The carbohydrate was converted to sugar using hydrolysis under hydrothermal condition in a small autoclave. Firstly, the investigation was carried out to find suitable conditions for hydrolysis before lipid extraction. The hydrolysis conditions were varied in terms of biomass loading (10-40 g/L), incubation temperature (180–220°C), reaction time (0.5-2 h), acid categories (HCl, HNO_3 and H_2SO_4) and acid concentration (0-2%wt). The maximum glucose content of *Scenedesmus* sp. and *Ankistrodesmus* sp. were 5.96 and 2.38 g/L, respectively, at 0.75%wt H_2SO_4, reaction temperature of 180°C and retention time 180 min. Acetic acid, furfural, HMF, levulinic acid and formic acid of both microalgae were generated and increased with acid concentration. At optimum condition, the total sugar acids were 0.63 g/L for *Scenedesmus* sp. and 1.30 g/L for *Ankistrodesmus* sp. The protein content in *Scenedesmus* sp. and *Ankistrodesmus* sp. were 5.68 and 5.22 g/L, respectively. The lipid contents of hydrolyzed residue were 13.78 (*Scenedesmus* sp.) and 24.88%wt (*Ankistrodesmus* sp.).

Secondly, the microalgae was extracted for their lipid contents where were 28.14%wt for *Scenedesmus* sp. and 14.9%wt for *Ankistrodesmus* sp. Hydrolysis of the lipid extracted residue gave the highest glucose contents of 6.08 and 1.89 g/L, respectively, at 0.5%wt H_2SO_4, reaction temperature of 200°C and retention time 120 min for *Scenedesmus* sp. and *Ankistrodesmus* sp. In this case, sugar acids were similar to hydrolysis before lipid extraction except that formic acid was not observed. At optimum condition, the total sugar acids were 0.99 g/L for *Scenedesmus* sp. and 1.16 g/L for *Ankistrodesmus* sp. The protein content in *Scenedesmus* sp. and *Ankistrodesmus* sp. were 5.93 and 5.85 g/L, respectively. Compared to hydrolysis before lipid extraction, the hydrolysis of solvent pretreated microalgae could decrease acid concentration and retention time.

Department: Chemical Engineering Student's Signature .....

Field of Study: Chemical Engineering Advisor's Signature .....

Academic Year: 2013

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CHAPTER I

INTRODUCTION

1.1 Motivations

Microalgae have received numerous attentions because of their many advantages. They have the potential to consume carbon dioxide which, in many instances, has been associated with the reduction of greenhouse gas emissions. In addition, they can remove nitrogen and phosphorus in polluted water (de-Bashan & Bashan, 2010). From chemical compositions of microalgae such as carbohydrate, protein and vitamin as well as antioxidants, algae extract can be used in versatile applications such as fertilization, animal feed and high-cost supplementary food. Nowadays, microalgae culture is mainly focused on biodiesel production due to their high lipid content, growth rate and requirement of smaller cultivation area (Chisti, 2007, Becker, 2007, Douskova *et al.*, 2009).

However, microalgae can suffer from harvesting and drying costs especially applications that need to use dry biomass. Recent development therefore concentrates much on reducing costs. One potential solution to this is to add value to the algal residue as most of the algal parts are usable in one form or another. For instance, Yang et al. (2011) showed that *Scenedesmus* residue after lipid extraction still contained 32.4% crude protein and 24.7% carbohydrate. Literature reported that enzymatic hydrolysis could convert carbohydrate into lipid-extracted microalgal residues (LMRs) into hydrogen. As much as 30 L H_2/kg dried LMRs could be obtained. Some reported the hydrolysis of carbohydrate in LMRs in *Chlorella* into methane with the yield in the range of 0.2–0.5 m^3/kg of dried LMRs (Morand & Briand, 1996, Briand & Morand, 1997). This approach can increase the value of microalgal residue and reduce chemical contaminated waste.

The efficient conversion technique for carbohydrate from biomass is hydrolysis. There are various hydrolyzed techniques such as enzymatic hydrolysis, catalytic hydrolysis (Zhou *et al.*, 2011, Laopaiboon *et al.*, 2010) and hydrothermal hydrolysis. Although the use of acid or enzyme can help improve hydrolysis yield (Singh & Saldaña, 2011, Jeong *et al.*, 2012, Gao *et al.*, 2012), they require additional chemicals and in some cases this necessitated an additional washing step. The hydrothermal technique has the advantage of being a more environmental friendly choice (no chemical additives), but has to pay a price of high energy consumption. It becomes the objectives of this work to optimize the hydrothermal hydrolysis of

carbohydrate in the algae and to examine the effect of acid in enhancing yield of sugar from carbohydrate in such a process.

1.2 Objectives

The objectives of this work are to investigate the efficiency of the hydrothermal hydrolysis reaction of microalgae carbohydrate before/after lipid extraction and to determine the effect of adding acids on such hydrothermal hydrolysis reaction.

1.3 Working Scopes

This work is carried out based on some limitations as follows:

1.3.1 Hydrothermal hydrolysis

- Microalgae (*Scenedesmus* sp. and *Ankistrodesmus* sp.) concentration ranges from 0.25-1.0 g in constant volume of water 25 mL
- Hydrolyzed temperature and time ranges from 180–220°C and 30–210 min respectively.

1.3.2 Acid catalyzed hydrothermal hydrolysis

- The acid categories used in hydrolysis are hydrochloric acid, sulfuric acid and nitric acid.
- The acid concentration ranges from 0.5%-2.0%wt

1.3.3 Lipid extraction

Soxhlet method for extraction use chloroform: methanol = 2:1 as solvent.

CHAPTER II

BACKGROUNDS AND LITERATURE REVIEW

2.1 Microalgae

2.1.1 General information

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms whose size range from micrometer to hundreds of micrometer. They are found in both freshwater and marine systems with diversity of more than 50,000 existed species (Amos, 2004). Major compositions of microalgae basically include protein, carbohydrate, lipid, fiber and ash which are different from species to species as listed in Table 2.1. Applications of microalgae have been proposed based on the compositions in cell used as raw material for biodiesel, fertilization, supplement food and animal feed.

Table 2.1 General composition of different microalgae (% of dry matter) (Becker, 2007)

Commodity	Protein	Carbohydrate	Lipid
Anabaena cylindrical	43–56	25–30	4–7
Chlamydomonas rheinhardii	48	17	21
Chlorella vulgaris	51–58	12–17	14–22
Dunaliella salina	57	32	6
Haematococcus pulvialis	29	19	28-24
Porphyridium cruentum	28–39	40–57	9–14
Scenedesmus obliquus	50–56	10–17	12–14
Spirulina maxima	60–71	13–16	6–7
Synechococcus sp.	63	15	11

2.1.2 Utilization of products from microalgae

The idea of utilizing microalgal lipid as an optional biodiesel feedstock started from the fact that most microalganisms contain some amount of lipid. However, the production of energy from microalgae is still uneconomical. Step of the extraction of oil from the algal cells consumes unreasonably high energy, leading biodiesel production from this source economically unfeasible (Chisti, 2007). However, microalgae have always been used as a nutritional supplement or represent a source of natural food colorants (Borowitzka, 1999). For example, *Chlorella* is found containing a great amount of carbohydrate that can apply for supplementary food (Zhao *et al.*, 2013). *D. salina* can produce β-carotene up to 15% of its dry weight (Macías-Sánchez *et al.*, 2009). Carotenoids have the ability to act as provitamin A, (García-González *et al.*, 2005). Astaxanthin is found most abundant in *Haematococcus pluvialis* (Hejazi & Wijffels, 2004). Microalgae also provide high-value polyunsaturated fatty acids, e.g. omega 3 in the forms of DHA and EPA, and omega 6 in the forms of GLA.

Table 2.2 Utilization of products from microalgae

Author	Purpose of study	Strain	Method	Result
Macías-Sáncheza (2009)	Carotenoids	Dry powder of *Dunaliella salina*	Supercritical CO_2 extraction	Highest yield of carotenoids achieved 15 % dry algae.
Tang et al. (2011)	Lipids and enrichment of DHA	Dry powder of *Schizochytrium limacinum*	Supercritical CO_2 extraction	Highest yield achieved 33.9% of lipid and 27.5% of DHA content.
Guili et al. (2013)	Carbohydrates	Dry powder of *Chlorella* sp.	Ultrasonic assisted extraction	Highest yield of glucose was 27% dry algae.
Sari et al. (2013)	Protein	Dry powder of *Chlorella fusca*	Enzymatic hydrolysis (Protex)	Highest yield of protein was 78% (w/w).
Wang et al. (2012b)	Astaxanthin	Dry powder of *Haematococcus pluvialis*	Supercritical CO_2 extraction	Highest yield of astaxanthin was 87.42%wt.

2.2 Microalgal constituents

2.2.1 Carbohydrate

A carbohydrate is an organic compound that consists of carbon, hydrogen, and oxygen, formed in general formula: $C_x(H_2O)_y$. They are classified according to the number of single carbohydrate molecules that are divided into three chemical groupings: *Monosaccharides*, *Disaccharides* and *Polysaccharides* (see Figure 2.1).

Carbohydrates in microlagae can be found in the form of starch, glucose, sugars and other polysaccharides that can be converted into reducing sugar or fermented into bioethanol. *Chlorella*, *Dunaliella*, *Chlamydomonas*, *Scenedesmus*, *Spirulina* are known to contain a large amount (>50% of the dry weight) of starch and glycogen, useful as raw materials for ethanol production (Hirayama *et al.*, 1996, Harun *et al.*, 2010). In a study of Choi et al. (2010) reported that *Chlamydomonas* could accommodate around 60% carbohydrates (44% of which was starch) which was hydrolyzed and converted into glucose by S. cerevisiae. The result was 44.7% glucose, while the total compositions of the other monosaccharide were 7.3%. The algal biomass is cheaper and contains less intracellular glucose than other biomass sources.

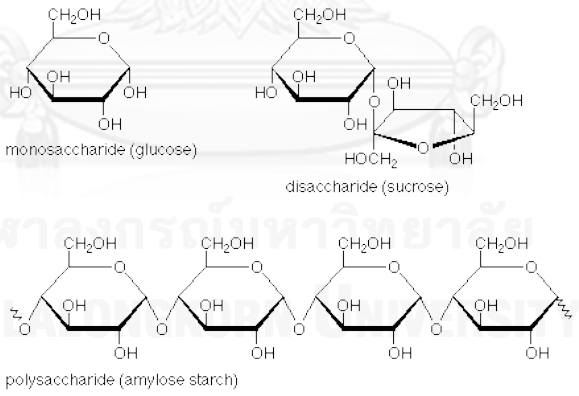

Figure 2.1 Structural molecule of carbohydrate

Ref: http://chemistry2.csudh.edu/rpendarvis/monosacch.html

2.2.2 Lipid

Simple lipid includes fats, oils and waxes. Both fats and oils, also called glyceride, can be formed from a glycerol and fatty acids. Fatty acids may be classified into 2 groups. One is saturated fatty acids, all of carbon atoms in R groups are attached with only single bonds. The other is unsaturated fatty acids that contain carbon atom bonded with at least one double or triple bond. Waxes are compounds of large molecules of fats and alcohols.

As aforementioned, the average lipid content of algal cells varies between 4% and 30% but can reach 90% of dry weight under certain conditions (Metting, 1996). Volkman et al. (1989) studied the composition of lipid extracts from microalgae and found that microalgae contained amounts of triglycerides and free fatty acids that can turn in to biodiesel. Biodiesel is a product from alcohol and oil reaction, called esterification (Figure 2.2). Unfortunately, biodiesel from microalgae do not only contain the target compound but they also contain unneeded forms of lipids and other impurities that decrease the yield of biodiesel conversion (Canakci & Van Gerpen, 1999).

Soxhlet (Figure 2.3) is one of various techniques of solvent extraction. The solid sample is extracted with an always-fresh-solvent repeatedly so the result from soxhlet method must be nearly 100% recovery. In industrial scale, n-hexane is the most commonly used solvent which is mixed with microalgae biomass. The oil dissolves in n-hexane and the biomass can be filtered out from the medium through distillation. The inherent disadvantage of this method is that solvent extraction may deem the residual biomass unfit for use as animal feed.

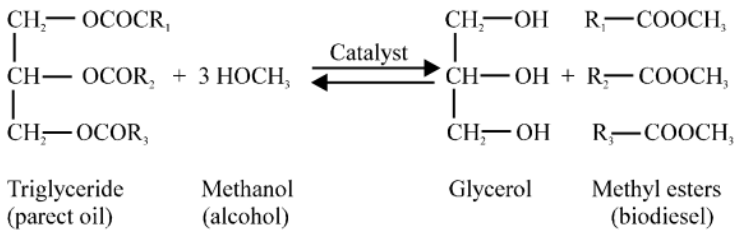

Figure 2.2 Transesterification pathway

Ref: http://econuz.com/page/7/

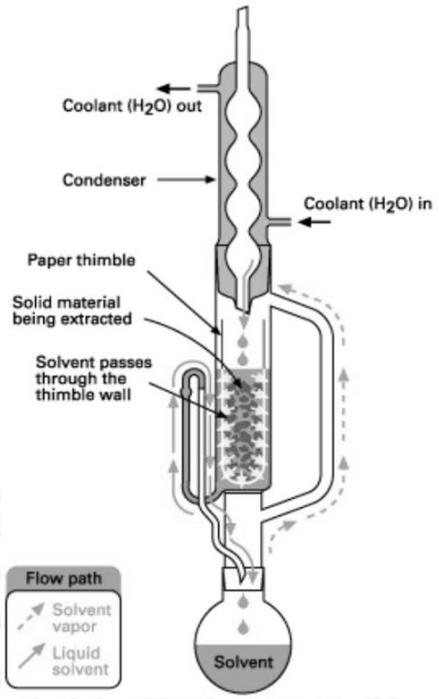

Figure 2.3 Soxhlet Extractor

Ref: http://ainstrumental.wikispaces.com/EQUIPO+SOXHLET

2.3 Hydrolysis

In general term, hydrolysis is a chemical transformation of organic molecule. Hydrolysis is a step in the degradation of a substance such as hydrolyzed cellulose to be a glucose or protein to be amino acid. From Equation 2.1, AB reacts with water, resulting in breakage of the covalent bond between A and B and the formation of a new covalent bond of A with H and B with OH. This explain why it is called hydrolysis, meaning splitting by water.

$$AB + \mathrm{HOH} \rightleftharpoons A\mathrm{H} + B\mathrm{OH}. \quad (2.1)$$

The hydrolysis reaction rate depends on amount of hydrogen ions, H+ and hydroxide ions, OH- that will be replace in the reactant. Reaction rate can increase by raising the cleaving water rate or adding catalysts that release hydrogen or hydroxide ions. Various modified hydrolysis are described as follows.

2.3.1 Enzymatic hydrolysis

Enzymes increase rate of reaction by lowering activation energy for a specific reaction as shown in Figure 2.4. Advantages of this method are that it can be operated at low temperature, high selectivity and low side products. However, the limitations of using enzymes for hydrolysis are expensive and long reaction time (Moreschi *et al.*, 2004). Their activity can easily be inhibited by the products of the reaction (Himmel *et al.*, 1999). In addition, without proper pretreatment, the hydrolysis might require high loading of enzymes (Harun & Danquah, 2011, Choi et al., 2010). To solve these problems, a new technique is developed such as immobilization of enzyme on membrane for reusability. Fu *et al.* (2010) and Cheng *et al*. (2010) presented that the immobilized enzyme on nanofibrous membrane could be reused but the hydrolysis yield decreased in recycling membrane after five cycles.

2.3.2 Acid catalyzed hydrolysis

Acid can dissolve in water and release hydrogen ions breaking covalent bond between monomer. The mechanism of acid catalyzed hydrolysis is shown in Figure 2.4, which presents that a hydrogen ion from acid reacts with substrate before water. This indicates that hydrogen ion is active in binding with substrate. The hydrolysis reaction rate depends on amount of hydrogen ion in each acid. From Table 2.3 (Bobleter, 1994), pK_a values show the efficiency of releasing hydrogen ions for each acid. HCl has the most negative pKa meaning that it is suitable as acid catalyst in hydrolysis (Neureiter *et al.*, 2004, Laopaiboon et al., 2010, Zhou et al., 2011). On the other hand, CH_3COOH is the poorest hydrolytic catalyst. The hydrolytic activity of most acid solutions, are also indicated by their pH value which can be increased by

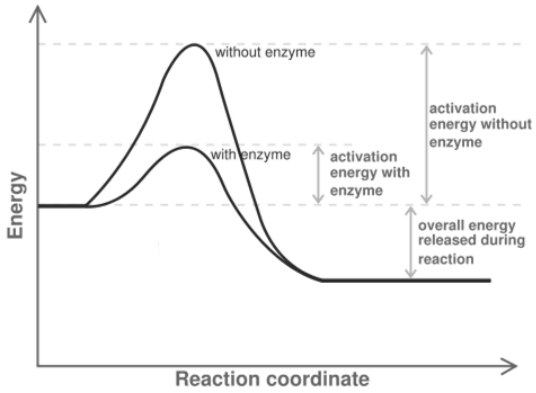

Figure 2.4 Energies of the stages of chemical reaction without enzyme compared with the reaction with enzyme (Ref. http://en.wikipedia.org/wiki/Enzyme)

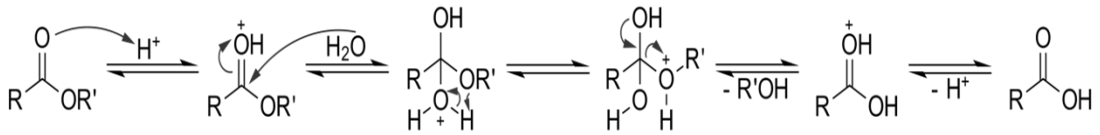

Figure 2.5 Acid catalyzed hydrolysis

Ref: http://en.wikipedia.org/wiki/File:Fischer_Esterification-Hydrolysis_Equilibrium.png

Table 2.3 pK_a values of acids (Ortwin, 1984)

Acid	HCl	H_2SO_4	HNO_3	CF_3COOH	H_3PO_4	HCOOH	CH_3COOH
pK_a	-6	-3	- 1.32	0.23	1.96	3.7	4.8

raising the temperature. In the same way, Mao *et al.* (2012) and Park *et al.* (2012) reported that the acetic acid at low temperature reaction was poor, but this was enhanced with temperature. Disadvantages of this method are that products and acid solution are homogeneous so an additional expense is needed for separating products out of acid solution. In addition, the acid waste solution is environmentally unfriendly.

2.3.3 Solid-acid catalytic hydrolysis

Solid-acid catalysts can be prepared by physical or chemical covalent bonding to attach the active molecule to a support. They are categorized by their Brønsted or Lewis acidity, the strength and number of these sites, and the morphology of the supports (e.g. surface area, pore size). To obtain high selectivity of the desired products in a hydrolysis reaction all of these properties must be considered. The pore size solid catalyst has influence on diffusion rate in liquid phase reaction. High surface area would increase possibility of contacting between substrate and catalyst. For example, SiO_2 has the highest surface area compared with Al_2O_3, TiO_2 and ZrO_2 and could accelerate the hydrolysis of cellulose into glucose selectively (Wang et al., 2012a). Development of heterogeneous acid catalysts was developed to replace homogeneous catalysts because it can be recovered and friendly to environment. Lou et al. (2008) and Wang *et al.* (2012a) revealed that proper catalysts could still provide high catalytic activity even after 50 cycles of reuse and thus showed excellent stability.

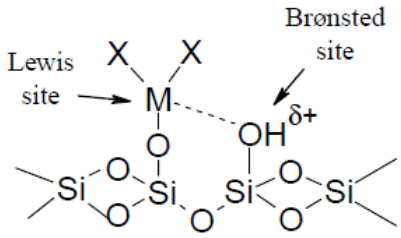

Figure 2.6 Brønsted and Lewis acidity on a silica support.

(Karen and James, 2000)

2.3.4 Hydrothermal hydrolysis

This method of hydrolysis uses water at temperatures higher than 150°C and pressures from 0.1 up to 25 MPa. Water at such conditions is also known as subcritical water or water at temperatures below its critical point and under pressurized conditions. At this condition, water has several very interesting properties. Subcritical water is low viscosity and high solubility of organic substances, which make an excellent medium for fast, homogeneous and efficient reactions. Singh *et al.* (2011) found that subcritical water at 160 to 180°C might be a good substitute to organic solvents to obtain phenolic compounds from biomass. From Table 2.4, as the dielectric constant decreases, the solubility of hydrophobic organic compound, such as free fatty acid, increases. The ionic product of subcritical water ($K_w = 10^{-12}$) is higher than normal and supercritical water ($K_w = 10^{-14}$ and $10^{-19.4}$) (Toor *et al.*, 2011) This property means that water could support the ionic or free-radical reaction because it has a greater ionic product: H_3O^+ and OH^-. Subcritical water has the properties as an acid or a basic catalyst (Kruse & Dinjus, 2007). Although this method proceeds slower than acid catalytic hydrolysis, it is interesting in terms of non-toxicity as severe catalyst can be negligible. The disadvantage of this method is that it consumes much higher energy than the others and this could make its economically impractical because of high temperature and long reaction time. To develop hydrothermal hydrolysis method, adding diluted acid catalyst is a good alternative. Shen *et al*. (2011), Mao *et al.* (2012), Jeong *et al.* (2012) and Sereewatthanawut *et al.* (2008) reported that using acid catalyst in reaction can decrease the reaction time. According to Park *et al*. (2012), acid efficiency in hydrothermal condition can increase 2 times.

Table 2.4 Properties of water in various conditions (Toor et al., 2011)

	Normal water	Subcritical water		Supercritical water	
Temp. (^{o}C)	25	250	350	400	400
Pressure (MPa)	0.1	5	25	25	50
Density (g cm^{-3})	1	0.8	0.6	0.17	0.58
Ionic product, K_w^*	10^{-14}	$10^{-11.2}$	10^{-12}	$10^{-19.4}$	$10^{-11.9}$
Dielectric constant (Fm^{-1})	78.5	27.1	14.07	5.9	10.5
Dynamic viscosity (mPa s)	0.89	0.11	0.064	0.03	0.07

Author	Source	Pretreatment	Hydrolysis method	Reactor	Products	Condition			Result
						Chemical	Temp.	Time	
Fu *et al.* (2010)	Microalgae: *Chlorella Sp.*	Dried by lyophilization for 24 h	Enzymatic hydrolysis	Polyacryloni-trile nanofibrous membranes	Reducing sugar	- Cellulase - pH 3.6, 4.5 and 7.6 - vary cell concentration 20, 30 and 40 g/L	50°C	0–70 h	- The highest hydrolysis yield 62% under condition 50°C, pH 4.6 and cell concentration 20 g/l. - The five times reuse of the immobilized enzyme decreased hydrolysis yield about 40%.
Harun *et al.* (2011)	Microalgae : *Chlorococu m humicola*	Freeze-drier	Enzymatic hydrolysis		Glucose	- Cellulase - pH 1.5–9 - vary enzyme to biomass ratios 0.02 - 0.1	28°C 48°C 60°C	0-72 h	- The highest glucose yield of 64.2% (w/w) was obtained at a temperature of 48 °C, pH 4.8, and enzyme to biomass ratios 0.1. - High loading enzyme increase % yield of glucose
Neureiter *et al.* (2004)	Silage and grass	Air dried	Acid catalyzed hydrolysis	Batch stainless steel reactor 20 L	Reducing sugar	- H_2SO_4 vary concentration 0.4–0.7 % - vary dry matter concentration 7–20%	160–180 °C	10-12 min	- The optimum condition of highest total sugar 21.13% is 0.5% H_2SO_4 and 13% biomass concentration at 70°C. - Untreated silage and grass give only poor yields when they are hydrolyzed with dilute sulfuric acid, because these materials have a high buffer capacity.
Laopaibo on *et al.* (2010)	Sugarcane bagasse	Dried at 90°C for 18 h	Acid catalyzed hydrolysis	Batch stainless steel reactor 15 mL	Xylose	HCl and H_2SO_4 vary acid concentration 0.5–5%, (v/v)	90–120°C	1–5 h	- The maximum yield of xylose is 22.59% obtained under the conditions of 0.5% of HCl at 100°C for 5 h - Xylose is a main substrate for enzymatic hydrolysis of lactic acid production by *Lactococcus lactis*

Table 2.5 Literature review on hydrolysis of short chain products from biomass

Author	Source	Pretreatment	Hydrolysis method	Reactor	Products	Condition			Result
						Chemical	Temp.	Time	
Zhou *et al.* (2012)	Microalgae: *Chlorella*	Dry	Acid catalyzed hydrolysis	Stainless steel cylindrical reactor 15 mL	Glucose	HCl, HCl + $MgCl_2$ 2.5% vary concentration of HCl 0.5, 1.5, 2%	180°C 120°C	10 min 60 min	- Total sugar yield of algae were increased by the addition of HCl 2% and $MgCl_2$ 2.5%, was obtained a sugar concentration 12% and a sugar recovery 83%.
Bootsma *et al.* (2008)	Oligo-saccharides	Slurried with 1 wt% silica gel for 1 h at 30°C	Solid acid catalyzed hydrothermal hydrolysis	Stainless steel batch reactor 300 mL	Glucose, xylose and arabinose	0.2 g of mesoporous silica catalysts	145-175°C	0-90 min	- The highest yield of sugar is at temperature 175°C but at different time glucose 65% at 75 min, xylose 60% at 60 min and arabinose 68% at 15 min.
Wang *et al.* (2012a)	Cellulose	Dry	Solid catalytic hydrothermal hydrolysis	Stainless steel autoclave	Glucose	- Al_2O_3 - TiO_2 - SiO_2 - ZrO_2 - SBA 15 - HZSM	170°C	12 h	- SiO_2 catalyst showed the high yield of glucose 50.1% and 73.3% of cellulose conversion.
Nagamori & Funazukuri (2004)	Starch from sweet potato	Dried at 333 K for 12 h	Hydrothermal hydrolysis	Stainless steel bomb-type batch reactor	Glucose		180-240°C	1-40 min	- Highest glucose yield was at 200°C for 30 min - When the heating conditions were very high temperature and long heating time, the glucose yield decreased

Table 2.5 Literature review on hydrolysis of short chain products from biomass

Author	Source	Pretreatment	Hydrolysis Method	Reactor	Products	Condition			Result
						Chemical	Temp.	Time	
Lamoolphak *et al.* (2006)	Yeast	Suspended in distilled water to 10%w/v	Hydrothermal hydrolysis	Stainless steel closed batch reactor 5 mL	Protein and amino acids		100–250°C	5–30 min	- Highest protein yield was at 250°C for 25 min. - Highest amino acids yield was at 100°C for 15 min.
Sereewatthanawut *et al.* (2008)	Rice bran	Dry	Hydrothermal hydrolysis	Stainless steel batch reactor 8.8 mL	Protein and amino acids		100-220°C	0–30 min	The highest yield of protein and amino acids were 219 and 8.0 mg/g of dry bran, and were obtained at temperature 200°C for 30 min that higher than obtained from conventional alkali hydrolysis.
Pushp and Marleny (2011)	Potato peel	Freeze dried for 3 days	Hydrothermal hydrolysis	Batch stainless steel reactor	Phenolic compounds		100-240°C	30-120 min	Subcritical water at 160 to 180 °C, 6 MPa and 60 min (yield of phenolic compounds 81.83 mg/100 g) might be a good substitute to organic solvents such as methanol and ethanol to obtain phenolic compounds from potato peel.
Gao *et al.* (2012)	Cellulose	Dried at 105°C	Hydrothermal hydrolysis	Stainless steel autoclave 500 mL	Gas and heavy oil		200–400°C	5–120 min	The hydrothermal condition should control temperature about 250°C and residence time more 2 h.

Table 2.5 Literature review on hydrolysis of short chain products from biomass

Author	Source	Pretreatment	Hydrolysis method	Reactor	Products	Condition			Result
						Chemical	Temp.	Time	
Shen *et al.* (2011)	Microalgae : *Spirulina*	Dried for 1 h at 105°C	Acid-catalyzed Hydrothermal hydrolysis	Small batch stainless steel reactor	Acetic acid	H_2O_2 supply is about 40-120%wt	220–320°C	20-120 s	A good acetic acid yield was 14.6% (on carbon based) at 300°C for 80 s with 100% H_2O_2 supply.
Park *et al.* (2012)	Seaweed: *Laminaria japonica*	Freeze-drier for 3 days	Acid catalyzed hydrothermal hydrolysis	Batch stainless steel reactor 200 cm^3	Reducing sugars	Constant 1% CH_3COOH concentration	200–260°C	28-42 min	The highest content of reducing sugar was 814.10 mg/100 g dried sample at 200°C, adding of 1% acetic acid
Mao *et al.* (2012)	Corncob	Air-dried	Acid catalyzed hydrothermal hydrolysis	Semi-batch tubing bomb reactor 2.1 L	Furfural	CH_3COOH 1-3% and co-acid $FeCl_3$	170–200°C	30 min	A maximum furfural yield, a lignin and cellulose removal rates obtained 67.89%, 54.79% and 25.71%, respectively, at 3% of acetic acid and 20 mol/m^3 of $FeCl_3$ and at reaction temperature 180 °C for 30 min
Jeong *et al.* (2012)	Microalgae: *G. amansii*	Pretreated at 150°C with 20%w/v H_2SO_4 for 15 min	Acid catalyzed hydrothermal hydrolysis	Sealed bomb tubular reactor	Glucose	- HCl - HNO_3 - H_3PO_4 - H_2SO_4 2.5-10%(w/v)	170–210°C	3 min	The highest glucose concentration was 9.81% by using H_2SO_4 10%w/v at 190°C for 3 min
Ma *et al.* (2012)	Cellulose	Dry	Acid catalyzed hydrothermal hydrolysis	Stainless steel reactor	Glucose	- HCl - $AlCl_3$ - $Al(OH)_3$ 0.005–0.07%	240–320°C	1–6 min	$AlCl_3$ were more effective in cellulose hydrolysis more than HCl and $Al(OH)_3$

Table 2.5 Literature review on hydrolysis of short chain products from biomass

CHAPTER III

MATERIALS AND METHODS

3.1 Flow chart of work

Experiments in this work could be described using the following flow chart. Detail of each section was delineated in this chapter.

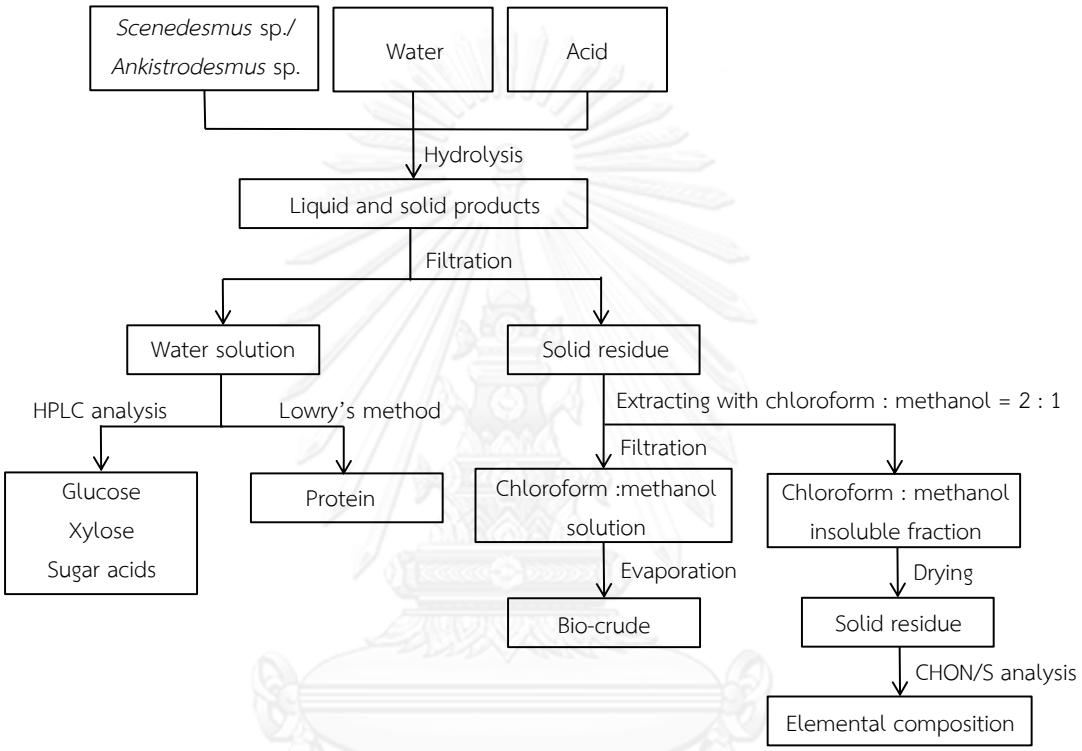

Figure 3.1 Outline of experiments and analysis in this work

3.2 Materials

Microalgae strains, *Scenedesmus* sp. and *Ankistrodesmus* sp. were chosen as a model study in this work. Fresh paste of *Scenedesmus* sp. and *Ankistrodesmus* sp. was obtained from the cultivation at the Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University, Bangkok, Thailand, where the culture was centrifuged at 4,500 rpm (3,398xg) for 10 min. The paste were frozen and then dried in a freeze-drier for 3 days. All samples were stored at 25°C until use. At the beginning, the dried algae were analyzed for carbon content including nitrogen and hydrogen with CHNS/O Analyzer (Perkin Elmer PE2400 Series II).

Figure 3.2 Small batch autoclave reactor

3.3 Hydrothermal hydrolysis

Hydrolysis experiment was conducted in pressurized autoclave reactor (stainless steel) with 30 mL working volume as depicted in Figure 3.2. The reactor was heated by an electric furnace (Furnace by CARBOLITE CTW 1100, England) at the rate of 15°C/min to a set-point reaction temperature.

3.3.1 Effect of microalgae dose

To investigate the effect of microalgae dose, the experiment was carried out as follows.

1. Mix 0.25 g dried microalgae with 25 mL DI water in 30 mL glass autoclave
2. Heat up the autoclave at 180°C for 2 hours in an electric furnace
3. Cool down the autoclave in 10°C ice bath for 30 min
4. Repeat Steps 1-3 with varying weights of dry algae, i.e. 0.5, 0.75 and 1.0 g

3.3.2 Effect of temperature and time of reaction

To investigate the effect of temperature and time of reaction, the experiment was carried out as follows.

1. Mix dried microalgae with the dose obtained from Section 3.3.1 with 25 mL DI water in 30 mL glass autoclave
2. Heat up the autoclave at 160°C for 30 min in electric furnace
3. Cool down the autoclave in 10°C ice bath for 30 min

4. Repeat Steps 1-3 with varying heated up temperatures, i.e. 180, 200 and 220°C
5. Repeat Steps 1-4 with varying heated up times, i.e. 60, 90, 120, 150, 180 and 210 min

3.4 Acid catalyzed hydrothermal hydrolysis

3.4.1 Effect of acid categories

To investigate the effect of acid categories, the experiment was carried out as follows.

1. Mix dried microalgae with the dose obtained from Section 3.3.1 with 25 mL of HCl 0.5%wt in the glass autoclave
2. Heat up the autoclave at optimized temperature from Section 3.3.2 in electric furnace
3. Cool down the autoclave in 10°C ice bath for 30 min
4. Repeat Steps 1-3 with varying acid categories, i.e. H_2SO_4 and HNO_3
5. Repeat Steps 1-4 with varying acid concentrations, i.e. 1.0, 1.5 and 2.0%wt

3.4.2 Effect of acid concentration, temperature and time of reaction

To investigate the effect of acid concentration, temperature and time of reaction, the experiment was carried out as follows.

1. Mix dried microalgae with the dose obtained from Section 3.3.1 with 25 mL of acid 0.25%wt as optimized in Section 3.4.1 in small glass of autoclave
2. Heat up the autoclave at 180°C for 150 min in electric furnace
3. Cool down the autoclave in 10°C ice bath for 30 min
4. Repeat Steps 1-3 with varying acid concentrations i.e. 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 and 2.0%wt
5. Repeat Steps 1-4 with varying heated up temperatures, i.e. 160, 180, 200 and 220°C
6. Repeat Steps 1-5 with varying heated up times, i.e. 150, 180 and 210 min

The mixtures from Sections 3.2-3.4 were filtrated through Whatman GF/C cellulose extraction thimble. The hydrolyzed microalgae were extracted for its lipid content using soxhlet extraction. The algal residual was analyzed for carbon and nitrogen contents using CHNS/O Analyzer and the filtered solvent was determined for its reducing sugar and protein using HPLC and Lowry's method, respectively.

3.5 Lipid extraction

Soxhlet extraction was treated here as a method that yields 100% of the lipid extract from microalgae. It was then carried out in order to determine the effect of hydrolysis conditions on the amount of lipid extracted and the period of extraction. The hydrolyzed microalgae from Section 3.2 was filtrated through cellulose thimble, assembled to a 250 round bottom flask containing 180 ml of chloroform (AR grade, RCI labscan Ltd.) and methanol (MeOH) (AR grade, Mallinckrodt Chemicals Inc.) with ratio of 2:1 (v/v). The solvent was heated to vaporize with the cycle rate of approximately 12 min/round and the extraction was carried out for 2 h or until the colorless extract was observed. To recover the lipid extracted, the solvent was subsequently evaporated using a rotary evaporator and the remaining lipid in the vessel was measured gravimetrically. The remaining sludge (extract) in cellulose thimble was dried at 30°C and analyzed for carbon and nitrogen contents using CHNS/O Analyzer.

In the step of lipid extraction followed by hydrolysis, the microalgae were extracted lipid by soxhlet extraction and dried at 80°C for 24 h before hydrolysis in Section 3.3-3.4.

3.6 Analyses

3.6.1 Anthrone-sulfuric method analysis for total carbohydrate

Total carbohydrate was determined using anthrone-sulfuric acid method (Sánchez Mirón *et al.*, 2002). The crude glucose extracts from 100 mg of algal biomass was mixed with 20%wt perchloric acid. The mixture was ultrasonicated for 2 h (40 kHz ultrasonic bath, Crest Ultrasonics (Malaysia) SDN.BHD) and left to hydrolyze for 12 h at room temperature. Thereafter, 1 mL extract was mixed and reacted with 5 mL anthrone solution [10 mg anthrone in 100 mL 72%wt sulfuric acid] in cooled water. The mixture in glass tube was placed into 100°C water bath for 12 min. After cooled to a temperature in cold water bath, absorbance of the mixture was detected at 630 nm using spectrophotometer (GENESYS 10 UV scanning, Thermo spectronic). The standard curve was obtained using different D-glucose concentrations at 10, 20, 40,

60, 80 and 100 µg/mL. Standard calibration curve of glucose was reported in Appendix A-1.1. The glucose concentration was expressed as following regression equation:

$$y = 0.327x \quad (R^2 = 0.999, P < 0.01) \tag{3.1}$$

where y (µg/mL) was absorbance of glucose solution at 630 nm

x was the glucose concentration.

Finally, for one sample, the total glucose yield (supernatant and sediment) was obtained per 100 g dry cell weight.

3.6.2 HPLC analysis for reducing sugar

The filtrates obtained from Sections 3.3-3.4 were analyzed for reducing sugars content by using a High performance liquid chromatography (HPLC). The HPLC (Shimadzu, LC-20AD, Japan) equipped with an autosampler. The column and detectors used for analysis were a Bio-Rad Aminex HPX-87H column (7.8 mm I.D. x 300 mm Length) and a refractive index detector (Shimadzu, RID-10A, Japan) respectively. The HPLC was operated at an oven temperature of 45°C with 0.6 mL/min flow of 5 mM sulfuric acid. The 10 µL of hydrolyzate filtered by membrane filter (0.45 µm) was injected. The content of total reducing sugars and acids were qualified by comparing the retention time with that of standard (Talukder *et al.*, 2012b).

3.6.3 Composition analysis

The carbon content including nitrogen and hydrogen of *Scenedesmus* sp. and *Ankistrodesmus* sp. were measured using CHNS/O Analyzer (Perkin Elmer PE2400 Series II). The carbon was analyzed for total organic carbon. The nitrogen content was analyzed for total protein by multiplied with the conversion factor of 4.44 (Whitney *et al.*, 2003). The moisture content and ash content in microalgae were determined using ASTM 3173-11 and ASTM 3174-12, respectively.

3.6.4 Lowry's method for protein

The protein content in the filtrates was analyzed by Lowry's method. Bovine serum albumin (BSA) was used as a standard. A standard curve was prepared by diluting BSA to 10, 20, 30, 40, 60, 80, 100, 150, 200 µg/50 mL. The 0.5 mL of filtrates and standards were added sodium hydroxide 1 N (0.5 mL) and hydrolyzed at 80°C for 20 minutes in boiling water bath. After that preparing reagent by mixing 1 mL of 1%

cupric sulfate, 1 mL of 2% sodium potassiumtartrate and 50 mL of 5% sodium carbonate. The 2.5 mL of reagent was added to the hydrolyzate. Then the mixture was placed at room temperature for 10 min prior to the addition of 0.5 mL of Folin-Ciocalteau reagent. Color was developed in 30 min at room temperature. Absorbance of the mixture was detected at 750 nm using spectrophotometer (GENESYS 10 UV scanning, Thermo spectronic). Standard calibration curve of protein was shown in Appendix A-3.

$$y = 0.4797x \quad (R^2 = 0.994, P < 0.01) \tag{3.2}$$

where y (μg/mL) was absorbance of protein solution at 750 nm

x was the protein concentration.

Finally, for one sample, the total protein yield was obtained for per 100 g dry cell weight.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Analysis of microalgae biomass

The ultimate analysis results of *Scenedesmus* sp. and *Ankistrodesmus* sp. biomass are shown in Table 4.1. *Scenedesmus* sp. contained more carbohydrate, less lipid and less protein than *Ankistrodesmus* sp. Interestingly, the total carbohydrate and protein in the microalgae biomass were not significant reduced after lipid extraction, i.e. the reductions of carbohydrate in *Scenedesmus* sp. and *Ankistrodesmus* sp. were only 0.74% and 5.29%, respectively. Broch et al. (2013) reported that the compositions such as carbohydrate and protein were not soluble in lipid-extracted solvents which could well describe the findings in this wok.

Table 4.1 Chemical composition of microalgal biomass

	Scenedesmus sp. (%wt)	*Ankistrodesmus* sp. (%wt)
Lipid	14.90	28.14
Ash	17.43	6.29
Moisture	1.76	1.53
Carbohydrate (Before lipid extraction)	37.14	30.59
Carbohydrate (After lipid extraction)	36.87	28.87
Protein (Before lipid extraction)	28.77	33.45
Protein (After lipid extraction)	28.33	33.44

4.2 Hydrothermal hydrolysis

4.2.1 Effect of Biomass concentration on sugar production

In this study, hydrothermal hydrolysis was carried out to determine the effect of microalgal concentration on glucose content at constant temperature of 180°C and constant reaction time of 120 min. This temperature was selected based on the reported optimal temperature range for hydrolysis of large complex carbohydrates such as cellulose and starch (Maki-Arvela *et al.*, 2011, Wyman *et al.*, 2005). The glucose compositions obtained during the hydrolysis of *Scenedesmus* sp. and *Ankistrodesmus* sp. biomass are shown in Figure 4.1. The results indicated that the highest glucose contents of *Scenedesmus* sp. (4.28%wt) and *Ankistrodesmus* sp.

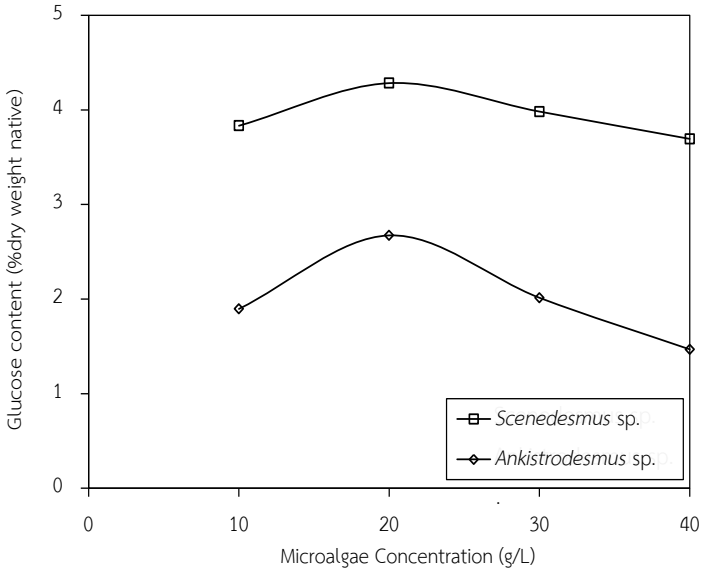

Figure 4.1 Effect of microalgae concentration on the glucose production in hydrothermal hydrolysis (reaction temperature 180°C and time 180 min, ash free condition)

(2.67%wt) were obtained at the microalgal concentration of 20 g/L. The glucose composition dropped when biomass loading increased. It was reported that, for a hot compress water system, the hydrolysis of carbohydrate must be catalyzed by proper ratio of H^+ and OH^- ions and biomass loading (Jena *et al.*, 2011). Too much biomass loading might lead to high mass transfer resistance which could slow down the hydrolysis rate (Chen *et al.*, 2013, Talukder et al., 2012b). For this case, the microalgae concentration of 20 g/L seemed to be the most suitable.

4.2.2 Effect of hydrolysis temperature and time

In order to investigate the effect of reaction temperature and time on glucose production at a constant biomass loading of 20 g/L, the glucose content was analyzed under hydrothermal hydrolysis temperature range of 160-220°C and reaction time of 20-210 min. The glucose contents of *Scenedesmus* sp. were generated as a function of reaction temperature and time as shown in Figure 4.2. At the lowest reaction temperature, 160°C, the glucose content increased gradually with reaction time and seemed to reach the maximum at 4.13%wt at 210 min. Increasing temperature to 180°C seemed to increase the glucose content to 4.65%wt (at reaction time 180 min). However, a further increase in temperature to 200°C saw a drop in glucose content to 4.30 (at 150 min) and 4.17%wt (at 180 min). Increasing reaction temperature above 200°C, the glucose content reduced.

Figure 4.3 shows the glucose contents of *Ankistrodesmus* sp. hydrolyzed at different reaction temperatures and times. A similar effect of temperature was observed for *Ankistrodesmus* sp. where the optimal reaction temperature and time were found at 180°C and 180 min. At this optimal, the highest glucose content of 3.18%wt was observed.

This finding could be explained as follows. Rising temperature increased the ionic products of water (H+ and OH-) (Toor et al., 2011). At the reaction temperature range of 160-180oC, the obtained hydrogen ion and energy in system was sufficient for hydrolysis of small carbohydrate such as starch. Increasing temperature within this temperature range helped enhance the production of glucose as observed from the results. However, temperatures range above 180oC, although gave a higher content of hydrogen ion, did not further increase the glucose concentration. This might be because this excess hydrogen ion further hydrolyzed glucose to smaller molecules such as hydroxymethylfurfural (HMF), levulinic acid and formic acid. Hence, the optimum reaction temperature and time were hereafter set out at 180oC and 180 min.

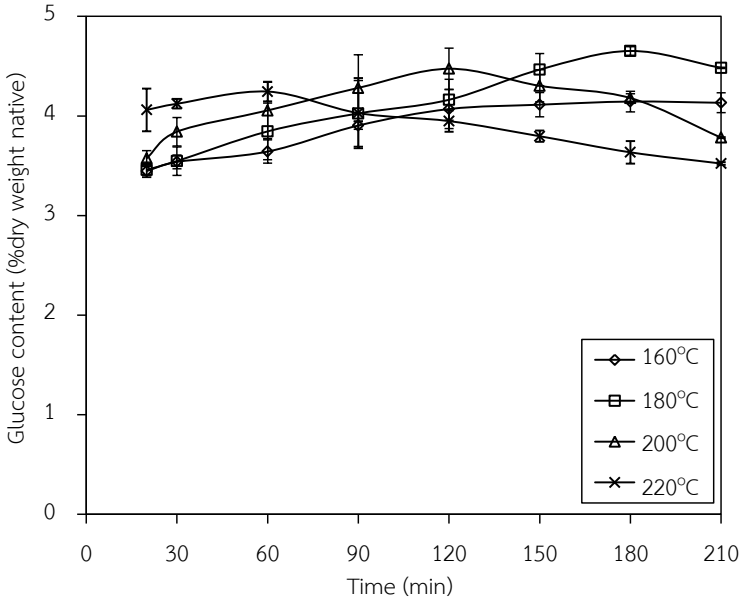

Figure 4.2 Effect of temperature and time on the glucose production in hydrothermal hydrolysis of *Scenedesmus* sp. (initial biomass loading of 20 g/L, ash free condition)

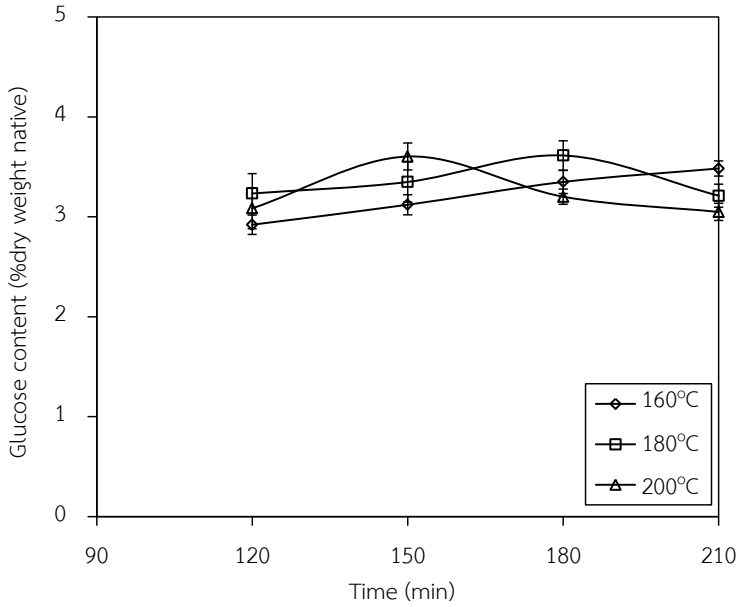

Figure 4.3 Effect of temperature and time on the glucose production in hydrothermal hydrolysis of *Ankistrodesmus* sp. (initial biomass loading of 20 g/L, ash free condition)

4.3 Acid hydrothermal hydrolysis

4.3.1 Effect of acid categories on sugar production

The acid hydrolysis of carbohydrate in microalgae was carried out using different acid reagents, such as HCl, H_2SO_4 and HNO_3. A constant reaction temperature of 180°C was used for 180 min on microalgal concentration of 20 g/L. The effects of various acid categories on reducing sugar content of *Scenedesmus* sp. and *Ankistrodesmus* sp. are illustrated in Figures 4.4 and 4.5, respectively. The glucose content decreased gradually as concentrations of HCl, H_2SO_4 and HNO_3 increased which could be due to the hydrolysis of the sugar itself as described in the previous section. The highest glucose contents of *Scenedesmus* sp. and *Ankistrodesmus* sp. were 28.04 and 10.95%wt, respectively, when using 0.5%wt of H_2SO_4. The xylose contents showed the opposite trend, i.e. the contents increased with the acid concentration. The maximum xylose contents of *Scenedesmus* sp. (2.22%wt) and *Ankistrodesmus* sp. (10.22%wt) occurred at H_2SO_4 2.0%wt. Frei and Preston (1964) reported that mannan and xylan were major polysaccharide in microalgae cell wall. Therefore adding more acid catalyst might result in the hydrolysis of such compounds giving xylose as a hydrolyzed product.

It was clear that the acid-catalyzed hydrolysis increased the glucose and xylose contents dramatically when compared to the results without acid as reported

in Section 4.2.2. The highest glucose content was 6 times higher than that obtained from typical hydrothermal reaction without acids whereas no xylose was detected in the case without acids. One potential reason for this is that acids provided extra hydrogen ion which could enhance the efficiency of breaking complex C-C, C-H bonds in carbohydrates.

The results of H_2SO_4-catalyzed hydrolysis showed the highest glucose and xylose contents except xylose content at 0.5%wt of HNO_3 of *Ankistrodesmus* sp. This effect indicated that the strong acid H_2SO_4 was active in breaking microalgal cell wall, resulting in more carbohydrate being released from the cell. It is worth noting that 0.5%wt (0.1219 M) H_2SO_4 had more or less the same molarity with 0.5%wt HNO_3 (0.1201 M) and lower molarity at than 0.5%wt HCl (0.1630 M). Despite so, H_2SO_4 was still the most efficient acid for such hydrolysis. This proves that H_2SO_4 exerted a strong influence in decreasing the activation energy of the hydrolysis reaction. Figures 4.6 and 4.7 demonstrated that the different acids with the same amount of proton did not provide the same hydrolysis power which indicates that the inorganic anion also had influence on the reaction. Sulfate seemed to be best in promoting the reaction and from the report of Roman and Winter (2004), it could be that sulfate ion helped decrease the activation energy of hydrolysis and therefore gave a better hydrolysis activity. Wu et al. (2012) also illustrated that sulfate was the most effective catalyst for reducing the activation energy compared with other acid anions such as chloride and nitrate. H_2SO_4 was therefore chosen as the suitable acid and used for the subsequent experiments.

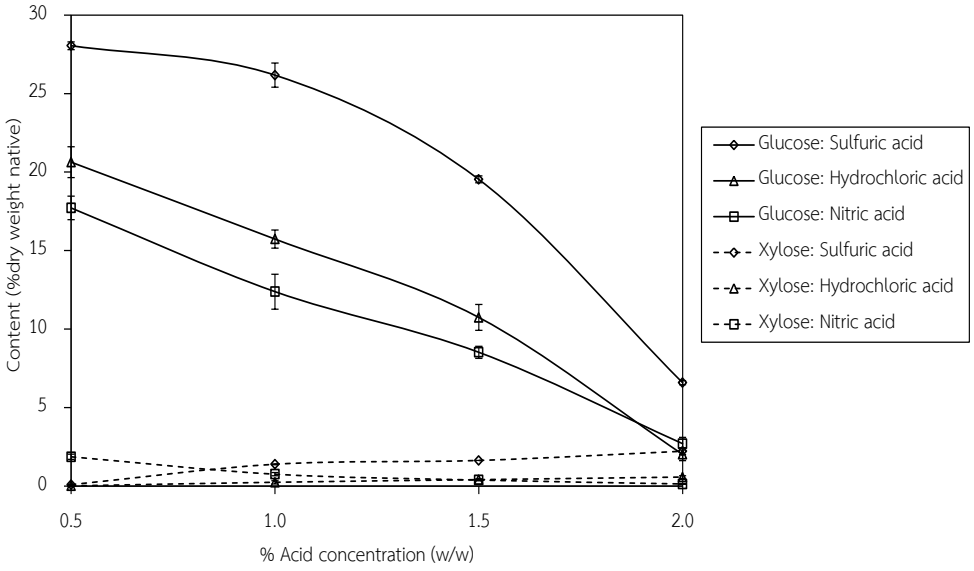

Figure 4.4 Effect of acid catalyst on the glucose production in acid-catalyzed hydrolysis of *Scenedesmus* sp. (initial biomass loading of 20 g/L, reaction temperature 180°C and time 180 min, ash free condition)

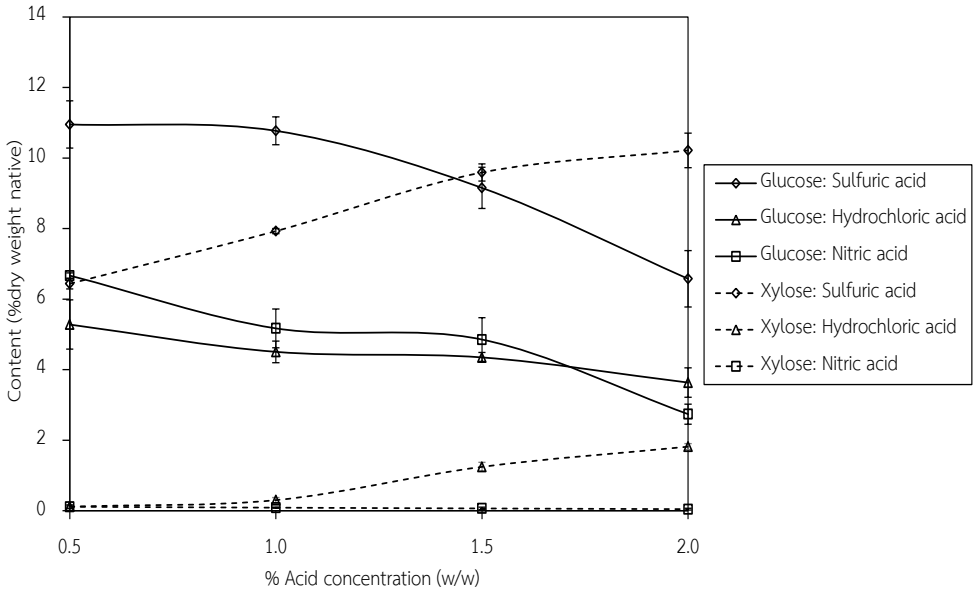

Figure 4.5 Effect of acid catalyst on the glucose production in acid-catalyzed hydrolysis of *Ankistrodesmus* sp. (initial biomass loading of 20 g/L, reaction temperature 180°C and time 180 min, ash free condition)

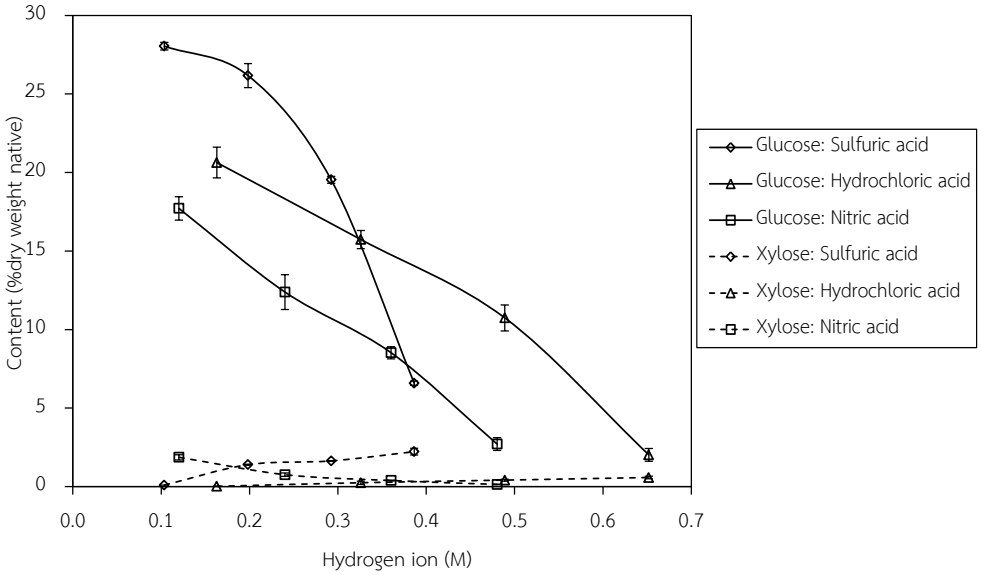

Figure 4.6 Effect of hydrogen ion on the glucose production in acid-catalyzed hydrolysis of *Scenedesmus* sp. (initial biomass loading of 20 g/L, reaction temperature 180°C and time 180 min, ash free condition)

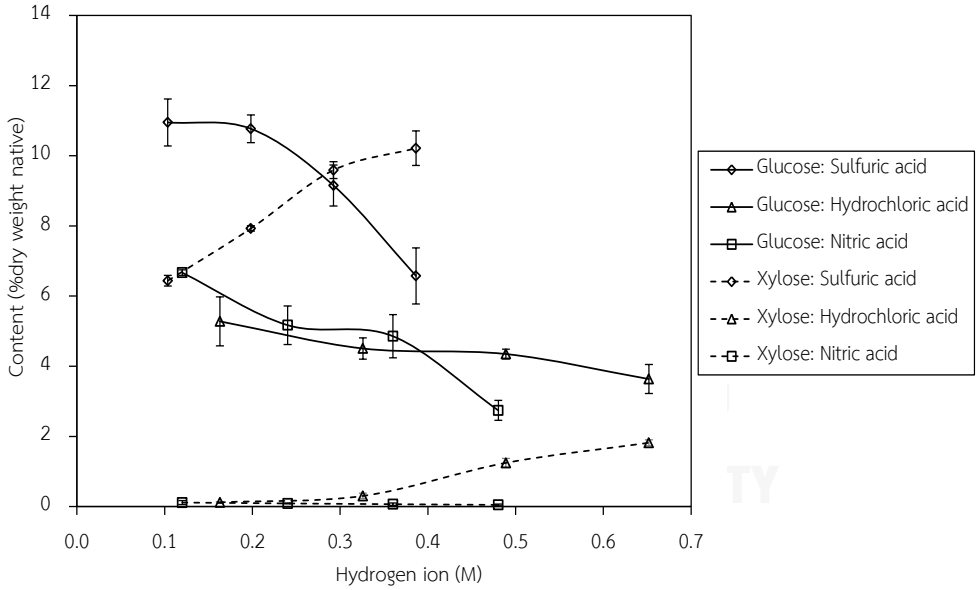

Figure 4.7 Effect of hydrogen ion on the glucose production in acid-catalyzed hydrolysis of *Ankistrodesmus* sp. (initial biomass loading of 20 g/L, reaction temperature 180°C and time 180 min, ash free condition)

4.3.2 Effect of H_2SO_4 concentration on sugar production

The analysis in this section was performed with a fixed microalgal concentration of 20 g/L, in an autoclave at a constant temperature 180°C and reaction time of 180 min, but with a variable H_2SO_4 concentration in the range of 0.25-2.0%wt. Figures 4.8 and 4.9 show the effects of acid concentration on sugar extraction efficiency from *Scenedesmus* sp. and *Ankistrodesmus* sp., respectively. The glucose contents increased steadily in the low H_2SO_4 concentration range (0-0.5%wt. The highest glucose contents were obtained at the acid concentration of 0.75%wt where the extracted glucose content from *Scenedesmus* sp. was 29.81 and from *Ankistrodesmus* sp. was 11.90%wt. A further increase in H_2SO_4 concentration to 2.0%wt observed a decline of the glucose content of both *Scenedesmus* sp. and *Ankistrodesmus* sp. to 6.57 and 6.47%wt, respectively.

The acid concentration had effects on xylose production. The xylose content in *Scenedesmus* sp. increased slightly, but the effect on *Ankistrodesmus* sp. was far more significant. The optimal acid concentration was found at 1.75%wt which gave the highest xylose content for both *Scenedesmus* sp. and *Ankistrodesmus* sp. (at 3.61 and 10.60%wt, respectively).

It is interesting to consider the different forms of sugar obtained from these two algal species. *Scenedesmus* sp. presented more glucose and less xylose, and the opposite was found for *Ankistrodesmus* sp. As glucose was reported to be derived from the hydrolysis of cellulose whereas xylose was from xylan (hemicellulose) (Brown, 1991, Dunstan *et al.*, 1992), It was anticipated that *Scenedesmus* sp. consisted of more cellulose than hemicellulose, and the opposite was expected to exist for *Ankistrodesmus* sp.

The results also revealed that adding more acid had an adverse effect on the sugar contents. At low acid concentration range (0.25-0.75%wt), sugar content increased with acid concentration because adding more acid gave more hydrogen ion required for the reaction with carbohydrates. On the other hand, at high acid concentration, excess hydrogen ion not only degraded carbohydrates, but it also did hydrolyze the sugar product resulting in a lower level of sugar being observed. These results agree well with the finding of Miranda et al. (2012).

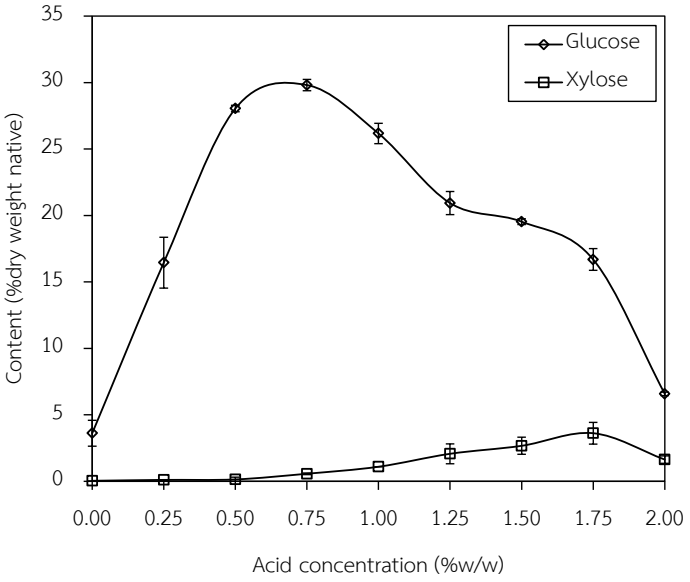

Figure 4.8 Effect of sulfuric acid concentration on the sugar production in hydrothermal hydrolysis of *Scenedesmus* sp. (initial biomass loading of 20 g/L, reaction temperature 180°C and time 180 min, ash free condition)

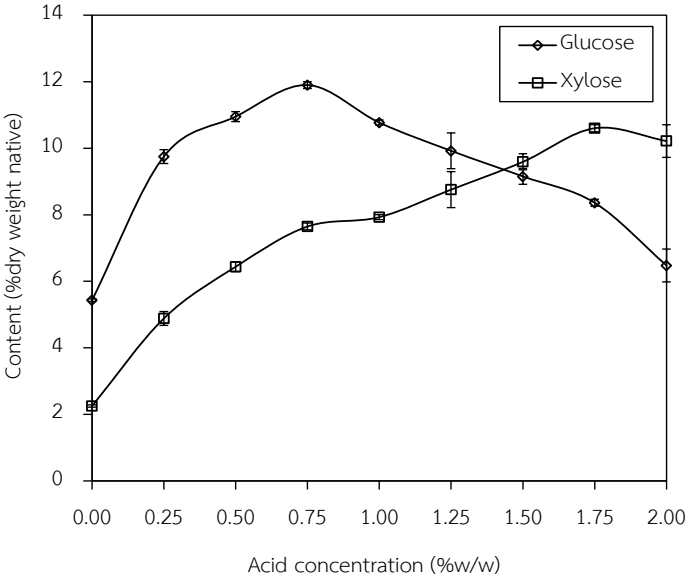

Figure 4.9 Effect of sulfuric acid concentration on the sugar production in hydrothermal hydrolysis of *Ankistrodesmus* sp. (initial biomass loading of 20 g/L, reaction temperature 180°C and time 180 min, ash free condition)

4.3.3 Sugar decomposed products from acid hydrothermal hydrolysis

Sugar obtained from the decomposition of algal biomass could be further decomposed in the hydrothermal reaction to what is so-called sugar acids and the results of H_2SO_4-catalyzed hydrothermal hydrolysis of *Scenedesmus* sp. and *Ankistrodesmus* sp. biomass with acid concentration 0.5-2.0%wt are shown in Figures 4.10 and 4.11. This is a simultaneous series reaction which took place straight after the hydrolysis of the algal biomass and therefore it was taken place at the constant reaction temperature of 180°C and the reaction time of 180 min. HPLC analysis demonstrates that the main sugar decomposition products during the hydrolysis were furfural, levulinic acid, formic acid, acetic acid and HMF. For *Scenedesmus* sp., at 0.25%wt H_2SO_4, the total sugar acid concentration was 0.53 g/L which consisted of HMF, acetic acid, formic acid, levulinic acid and furfural at 1.29, 0.80, 0.31, 0.19 and 0.07%wt, respectively. With the H_2SO_4 concentration increasing to 1.00%wt, the generations of formic acid and levulinic acid rose to 0.88 and 0.64%wt, respectively and the remaining glucose and xylose were 4.32 and 0.18 g/L. HMF did not increase greatly with increasing acid concentration because HMF had to be converted to formic acid and levulinic acid following the reaction pathway as suggested by Kupiainen *et al* (2011). At high H_2SO_4 concentration range (1.25-2.00%wt), the total sugar acid concentration increased to 0.91 g/L, and the final sugar content reduced to 1.32 g/L for glucose and 0.33 g/L for xylose. HMF, furfural, formic acid and levulinic acid were enhanced slightly to 1.16, 0.33, 1.11 and 0.79%wt, respectively. Unlike the other components, acetic acid content was maintained at 1.30%wt and did not seem to increase with an increase in the acid concentration. In addition, acetic acid seemed to reduce at a high range of H_2SO_4 (1.50%wt). This could be due to the fact that acetic acid was another intermediate which was generated and consumed to produce other products such as CH_4 and CO_2 (Rachmady and Vannice, 2000; Mora et al., 2009). However, the gaseous products could not be measured with the experimental setup employed in this work.

Considering *Ankistrodesmus* sp., the contents of HMF and furfural increased slightly with acid concentration whilst acetic acid maintained its concentration at 1.15%wt. As the H_2SO_4 concentration was enhanced from 0.25 to 1.00%wt, formic acid and levulinic acid contents increased as a result of acid-hydrolysis reaction significantly from 1.41 to 3.09 and 1.10 to 2.63%wt, respectively. The pattern of sugar derived products of *Ankistrodesmus* sp. was very similar to those of *Scenedesmus* sp.

Note that the final glucose and xylose concentrations obtained from the hydrolysis of *Ankistrodesmus* sp. were 1.23 g/L and 1.91 g/L, respectively (at 2.00%wt H_2SO_4).

Figures 4.10 and 4.11 reveal that formic and levulinic acids were generated approximately at the same point in time as the reduction in glucose in Figures 4.8-4.9. This suggested that formic acid and levulinic acid were the substances on the glucose decomposition pathway which was in good agreement with the reports from Peterson et al. (2008) and Jeong et al. (2012).

The amount of decomposed acids in hydrolyzate depended on the structure and quantity of carbohydrate in microalgae. *Scenedesmus* sp. gave more HMF, less levulinic acid and less formic acid because it had more carbohydrate. The main hydrogen ion would hydrolyze long chain complex carbohydrate, but there also existed a side reaction where hydrogen ion reacted with the glucose product and converted it to acids. It might be worth noting that *Scenedesmus* was described as having the strongest cell walls among most microalgae. Its cell wall was cellulose covered by a hemicellulose matrix which was difficult to hydrolyze (Lee *et al.*, 2010, Mussgnug *et al.*, 2010). *Ankistrodesmus* sp. provided the opposite trend to *Scenedesmus* sp. in that its hydrolysis product comprised less HMF, more levulinic acid and more formic acid. As a result of its low cellulose content, a larger quantity of hydrogen ion was remained from the hydrolysis of carbohydrates and this could decompose more glucose to formic acid and levulinic acid.

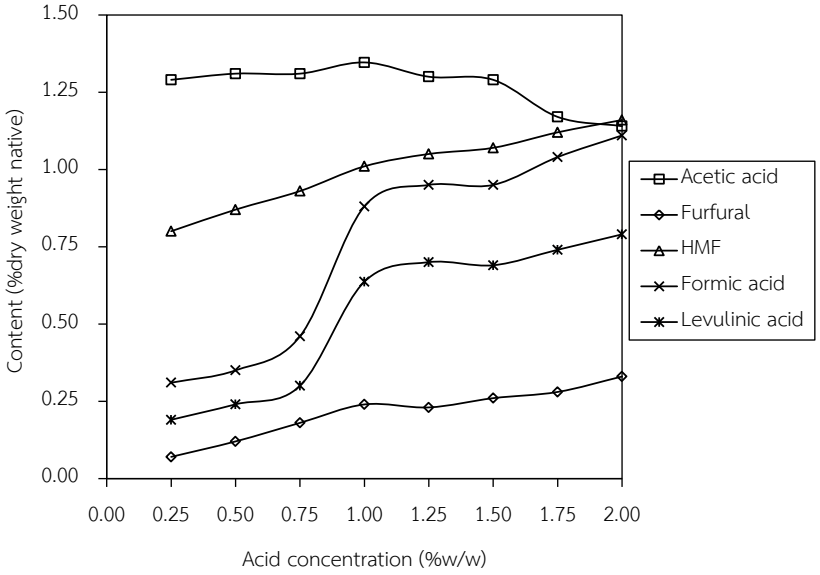

Figure 4.10 Behaviors of glucose decomposition in the hydrolyzate of *Scenedesmus* sp. (initial biomass of 20 g/L with different sulfuric acid concentration at reaction temperature 180°C and time 180 min, ash free condition)

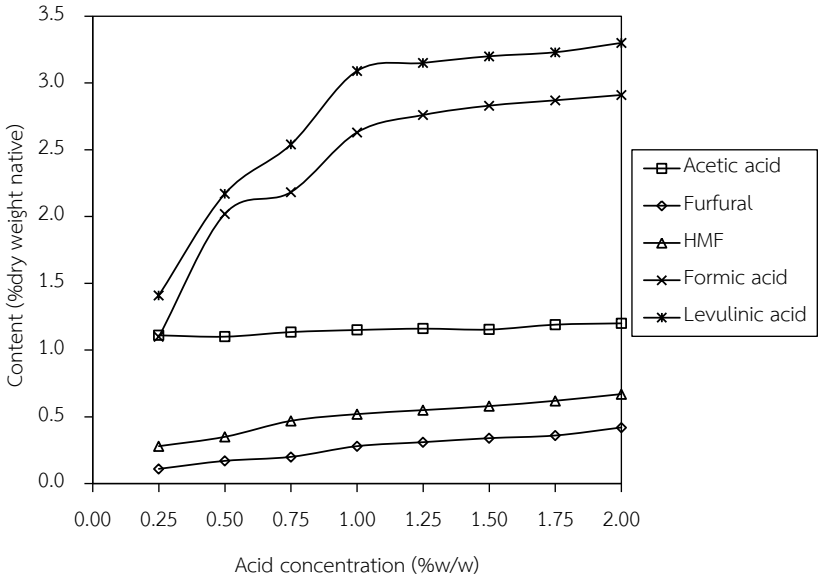

Figure 4.11 Behaviors of glucose decomposition in the hydrolyzate of *Ankistrodesmus* sp. (initial biomass concentration of 20 g/l with different sulfuric acid concentration at reaction temperature 180°C and time 180 min, ash free condition)

4.3.4 Lipid extraction post H_2SO_4-catalyzed hydrothermal hydrolysis

In this section, the hydrolyzed microalgae residue from Section 4.3.2 was subsequently extracted for lipid using a solvent mixture of chloroform and methanol at around 80°C. For comparison purpose, the lipid contents of hydrolyzed residues are shown in Figures 4.12 (*Scenedesmus* sp.) and 4.13 (*Ankistrodesmus* sp.).

For intact dry biomass of *Scenedesmus* sp. and *Ankistrodesmus* sp., the lipid contents were 14.90 and 28.14%wt, respectively. After hydrolysis (without acid), the lipid contents of hydrolyzed residues of *Scenedesmus* sp. and *Ankistrodesmus* sp. were 14.61 and 27.64%wt, respectively. This indicates that only about 2% of lipid was lost during the hydrothermal hydrolysis which was considered insignificant. In acid-catalyzed hydrothermal hydrolysis, the lipid content of hydrolyzed residue at 0.25%wt H_2SO_4 were 13.59 (*Scenedesmus* sp.) and 25.53%wt (*Ankistrodesmus* sp.). As H_2SO_4 concentration was increased, the amount of extracted lipid reduced, i.e. at 1.25%wt H_2SO_4, the extracted lipids of hydrolyzed *Scenedesmus* sp. and *Ankistrodesmus* sp. residues were reduced to 12.99 and 24.12%wt, respectively. The hydrolyzed residues with H_2SO_4 concentration higher than 1.25%wt became char-like (black powder after dry: labeled as “sample burnt” in Figures 4.12 and 4.13) which was not possible to enter a further lipid extraction step.

In conclusion, it was revealed in this section that the acid hydrothermal hydrolysis would at the end reduce the quantity of lipid that could be obtained from the algal biomass, there are some benefits inherited from such operation. First, as the hydrolysis weakened the cell structure (Talukder *et al.*, 2012a) and perhaps destroyed the integrity and opened up the cell structure, increasing the surface area for extraction (de Sena Cruz & Oliveira de Barros, 2004), the residue became more easily extracted by solvent extraction. As a result, extraction could be completed in a much less time duration than the virgin biomass. This helped save energy in the lipid extraction step. For example, dried *Scenedesmus* sp. biomass required the extraction time of 4 h but hydrolyzed residue reduced this extracted time to 2 h. Similarly, *Ankistrodesmus* sp. hydrolyzed residue could reduce extracted time from 5 to 3 h.

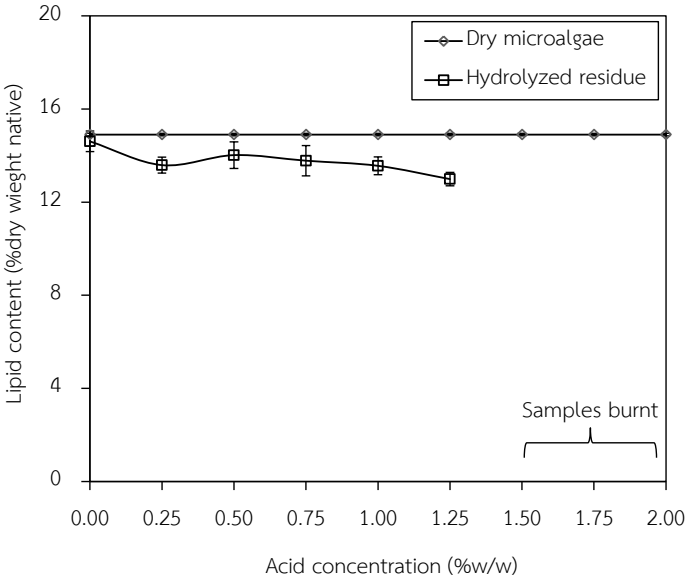

Figure 4.12 Effect of acid hydrolysis on lipid extraction from *Scenedesmus* sp. Hydrolysis conditions: microalgae 20 g/L, temperature 180°C, time 180 min; Extraction condition: 180 mL solvent (120 ml chloroform, 60 ml methanol)

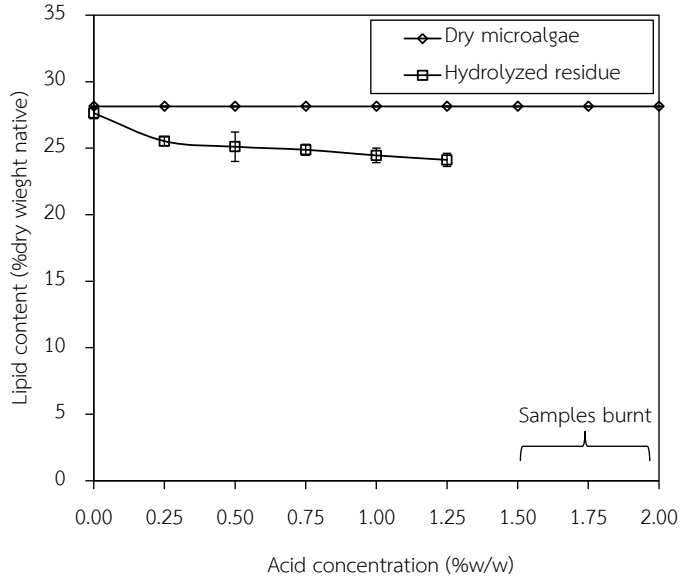

Figure 4.13 Effect of acid hydrolysis on lipid extraction from *Ankistrodesmus* sp. Hydrolysis conditions: microalgae 20 g/L, temperature 180°C, time 180 min; Extraction condition: 180 mL solvent (120 ml chloroform, 60 ml methanol)

4.4 Lipid extraction before hydrolysis

4.4.1 Lipid yield

Biomasses of *Ankistrodesmus* sp. and *Scenedesmus* sp. were extracted for their lipid contents using the solvent mixture between Chloroform and Methanol with the volume ratio of 2:1. *Scenedesmus* biomass gave the lipid yield of 14.9%wt whereas *Ankistrodesmus* yielded 28.14%wt. These were relatively larger than the yields from the hydrolysis residue, i.e. about 12.7% loss of lipid from *Ankistrodesmus* and 14.3% loss from *Scenedesmus*. This finding is contradicted to most reports from literature where more lipid was obtained from the hydrolysis algal residue (Fu et al., 2010). In this case, lipid molecules might have been destroyed by hydrolysis reactions or might have found its way out of the cell structure during the course of hydrolysis. The exact reason for this loss could not be concluded from the results of this work. The residues from lipid extraction of both algae entered the subsequent hydrolysis and the results are described as follows.

4.4.2 Effect of temperature, time and acid concentration on hydrolysis performance

Hydrolysis of carbohydrate remaining in the lipid-extracted microalgae biomass residues was carried out using H_2SO_4. The effects of temperature and time on glucose content in *Scenedesmus* sp. and *Ankistrodesmus* sp. are illustrated in Figures 4.14 and 4.15, respectively.

Scenedesmus sp. residue was hydrolyzed at 0.75% of H_2SO_4 at reaction temperature 180°C for 180 min, the optimum condition obtained from Section 4.3.2. Glucose was obtained at only 18.53%wt which was comparative lower than the hydrolysis of fresh alga (29.81%wt as reported in Section 4.3.2). The experimental conditions therefore were adjusted to find the new optimal. First, the reaction time was decreased to 120 min whilst the temperature was still maintained at 180°C. At this condition, glucose was increased with acid concentration and reached its maximum of 23.65%wt at 1.00%wt H_2SO_4. Raising temperature to 200°C at a constant reaction time of 120 min resulted in a dramatic increase in glucose content even at low H_2SO_4 concentration range. The highest glucose and xylose were 30.42 and 2.03%wt, respectively, at 0.50%wt H_2SO_4. This was virtually the same as the obtained glucose from the hydrolysis of fresh alga in Section 4.3.2. According to de Sena Cruz et al. (2013) and Girio et al. (2010), solvent and heat in extraction step might help break or weaken the microalgal cell wall and therefore allowed an easy further

extraction of other intracellular components. Hence, the pretreated biomass (from lipid extraction) required lower hydrolysis energy (Zhu *et al.*, 2009).

The pattern of sugar production of *Ankistrodesmus* sp. was very similar to *Scenedesmus* sp. The optimum condition of hydrolysis lipid extracted residue was 0.75%wt H_2SO_4 at reaction temperature of 200°C and time of 120 min. The highest glucose and xylose were 9.45 and 6.15%wt, respectively. When compared with the result in Section 4.3.2, the obtained glucose and xylose of *Ankistrodesmus* sp. decreased more than 20 and 40%, respectively. This loss might have occurred because the lipid extraction of *Ankistrodesmus* sp. required a much longer time than that of *Scenedesmus* sp. and this long pretreated time would increase the accessibility of H_2SO_4 to hydrolyze carbohydrates.

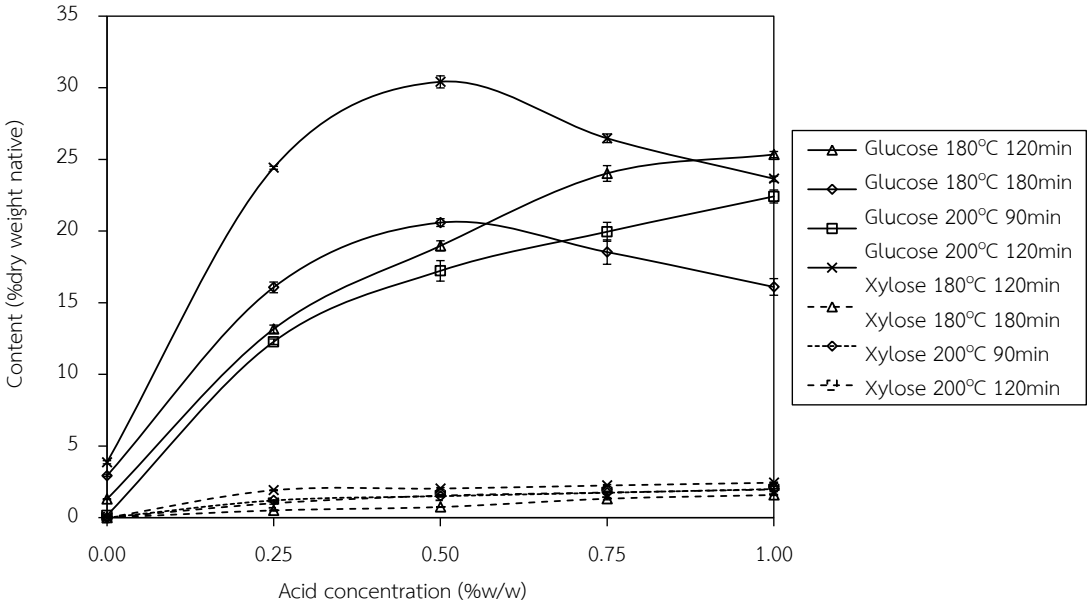

Figure 4.14 Effect of acid concentration, reaction temperature and reaction time of hydrolysis *Scenedesmus* sp. lipid extracted residue (initial biomass concentration of 20g/L, ash free condition)

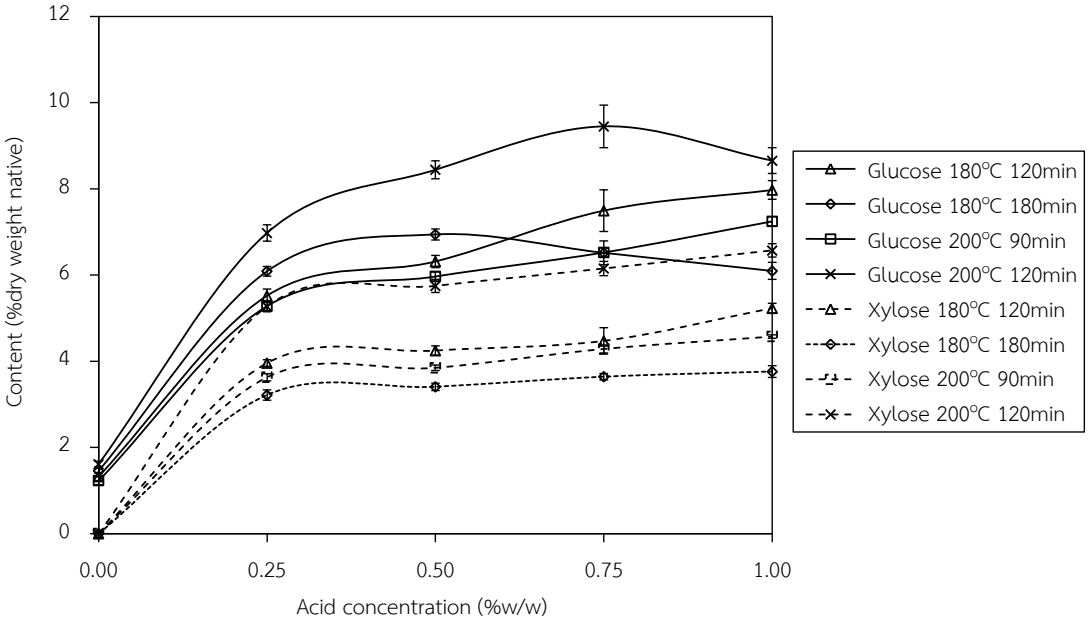

Figure 4.15 Effect of acid concentration, reaction temperature and reaction time of hydrolysis *Ankistrodesmus* sp. lipid extracted residue (initial biomass concentration of 20g/L, ash free condition)

4.4.3 Sugar decomposed products of lipid extracted microalgae residue from acid hydrothermal hydrolysis

Figures 4.16 and 4.17 illustrate the decomposition behaviors in H_2SO_4-catalyzed hydrothermal hydrolysis of lipid extracted residues of *Scenedesmus* sp. and *Ankistrodesmus* sp. with 0.25-1.00%wt of H_2SO_4 at constant temperature of 200°C and retention time of 120 min. For *Scenedesmus* sp. with 0.25%wt H_2SO_4 residue, acetic acid, furfural, HMF and levulinic acid were generated at 1.12, 0.19, 0.92 and 1.04%wt, respectively. Enhancing H_2SO_4 concentration to 1.00%wt, furfural, HMF and levulinic acid content increased slightly to 0.28, 1.18 and 1.33%wt, respectively, whereas acetic acid increased as much as two-fold to 2.25%wt. The behaviors of glucose decomposition of Ankistrodesmus sp. were similar to Scenedesmus sp. Furfural, HMF and levulinic acid increased with the acid concentration. Similarly, acetic acid increased 1.5 times from 1.33 to 1.99%wt. Note that large amounts of HMF and levulinic acid were already obtained at low H_2SO_4 concentration range (0.25-0.75%wt) when compared with the hydrolysis of fresh algal biomass where these sugar-decomposed products were obtained only at high acid concentration range (Section 4.3.3).

Similar to the findings in Section 4.3.3, the hydrolysis of lipid extracted residue of *Scenedesmus* sp. yielded more HMF and less levulinic acid when compared with the biomass of *Ankistrodesmus* sp. This could be attributed to the fact that *Scenedesmus* sp. had more cellulose and less hemicellulose than *Ankistrodesmus* sp. as previously described. It is interesting to observe that formic acid was not appeared from both algae. This could be due to the reaction between formic acid and contaminated methanol from lipid extraction step which gave rise to the formation of acetic acid (Indu *et al.*, 1993) as observed from the results. On the other hand, Deng et al. (2010) suggested that methanol (which could still reside in the mixture from the lipid extraction step) might be transformed into functional groups of carbohydrate, and when this carbohydrate was hydrolyzed, it would give methyl glucosides. Methyl glucosides were further decomposed to methyl levulinate and acetic acid and no formic acid was formed. Either of these or both mechanisms could be the reason for the absence of formic acid in this case.

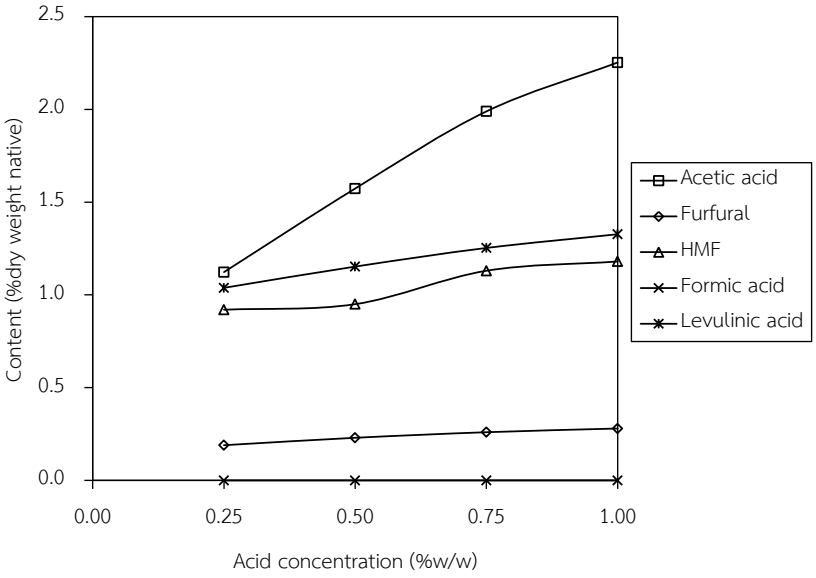

Figure 4.16 Behaviors of glucose decomposition in the hydrolyzate of *Scenedesmus* sp. lipid extracted residue (initial biomass concentration of 20 g/L with different H_2SO_4 concentration at reaction temperature 200°C and time 120 min, ash free condition)

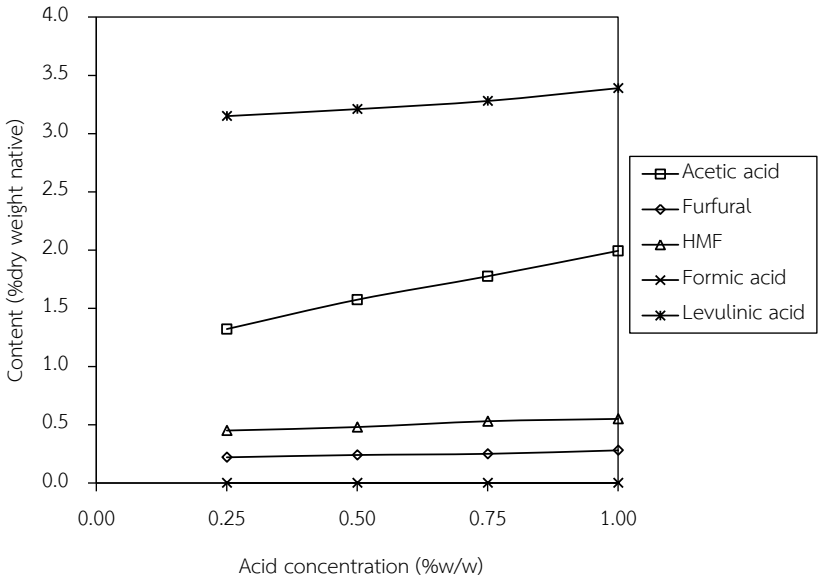

Figure 4.17 Behaviors of glucose decomposition in the hydrolyzate of *Ankistrodesmus* sp. lipid extracted residue (initial biomass concentration of 20 g/L with different H_2SO_4 concentration at reaction temperature 200°C and time 120 min, ash free condition)

CHAPTER 5

CONCLUSIONS

5.1 Hydrolysis and lipid extraction products distribution

This section summarizes the overall products derived from *Scenedesmus* sp. and *Ankistrodesmus* sp. which were extracted for their biochemical contents using a series of hydrolysis and lipid extraction steps. The results are given in Figures 5.1 and 5.2. It is worth reminded that xylose, glucose, protein and sugar acids were obtained as hydrolyzate products, whereas lipid was extracted with a mixture of chloroform and methanol. At the end of the experiment, the algal residue was burnt in furnace at 650°C for 6 h and the burnt remnant was labeled as ash which was insoluble inorganic compounds. The total weight loss when compared between the initial and final weights was considered as unknown comprising organic and inorganic volatile components.

The highest amount of product in hydrolyzate was protein content. Almost all protein in microalgae was hydrolyzed to soluble product. On the other hand, only 80% of the total carbohydrate in microalgae was decomposed, indicating the existence of organic content that cannot be decomposed or hydrolyzed even at higher temperatures. From observation, lipid-rich microalgae often had low reducing sugar content and vice versa. *Scenedesmus* sp. gave low lipid content of 14-16%wt, therefore it presented high sugars content of 24-27%wt. In contrast, *Ankistrodesmus* sp., offered high lipid content of 23-29%wt, hence, a low reducing sugars of only 7-18%wt.

Overall, the optimal condition of hydrolysis dried microalgae was 0.75%wt H_2SO_4 at reaction temperature of 180°C and reaction time of 180 min. In hydrolysis lipid extracted residue, the best condition was 0.50-0.75%wt at reaction temperature of 200°C and reaction time of 120 min. Hydrolysis of lipid extracted residue often showed more sugar acids and protein.

Much of the ash content presented in the starting biomass was not seen in the unreacted content, indicating that the hydrolysis process was effective in extracting some of inorganic compound into the aqueous phase (Daneshvar *et al.*, 2011). For example, the ash content of dried *Scenedesmus* sp. biomass was 17.43 %wt and after acid catalyzed hydrolysis, the ash content deceased to 8.48%wt.

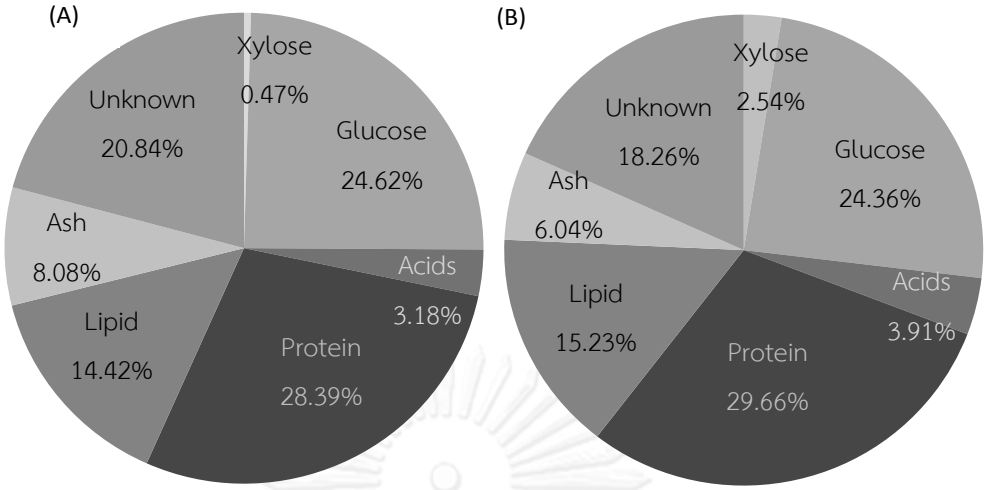

Figure 5.1 *Scenedesmus* sp. products distribution: (A) Hydrolysis (H_2SO_4 0.75%wt 180°C 180 min) before lipid extraction, (B) Lipid extraction before hydrolysis (H_2SO_4 0.50%wt 200°C 120min)

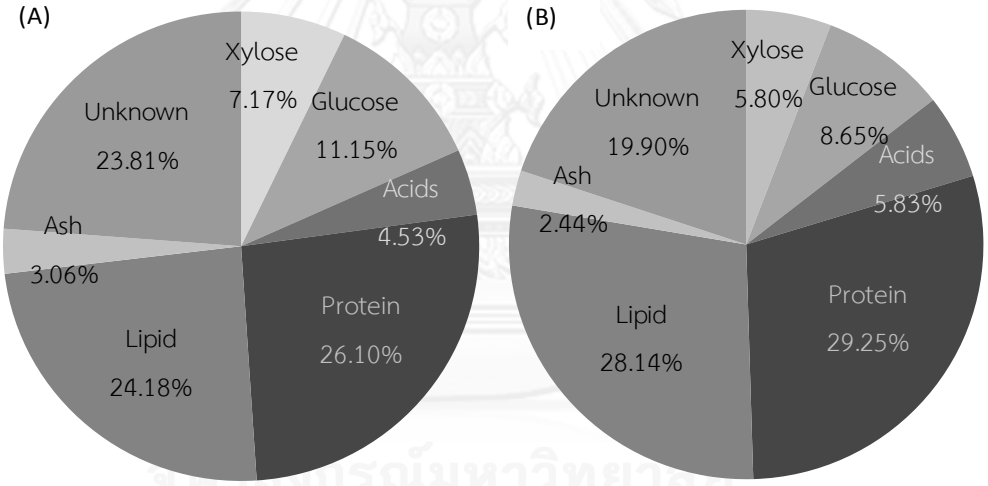

Figure 5.2 *Ankistrodesmus* sp. products distribution: (A) Hydrolysis (H_2SO_4 0.75%wt 180°C 180 min) before lipid extraction, (B) Lipid extraction before hydrolysis (H_2SO_4 0.50%wt 200°C 120min)

5.2 Economical analysis

The analysis of economics for the extraction processes is given in the last section of this work to provide addition information regarding the costs of the two extraction options, i.e. (a) hydrolysis before lipid extraction, and (b) lipid extraction before hydrolysis. The economic analysis was based on 1 batch of experiment (0.5 g of the fresh algae). From Table 4.2, post hydrolysis could diminish electric cost, especially in the hydrolysis step, more than 50%, and reduce the amount of acid

from 0.75 to 0.50%wt. Decreasing the amount of acid had an advantage as a smaller amount of acidic wastewater would be generated which could lower the cost of waste management (which is not included in this work). Hence, the solvent pretreated microalgae could save the cost more than 16%.

It is noted further that, for all obtained products, the protein has the highest value, therefore, high protein residue from the post hydrolysis method seemed to give a more attractive return. However, the cost of the production as proposed in this work (and with the prices as quoted at the time that this work was conducted) is still far too high for the profitable investment, therefore it is not recommended at this stage to carry out such a process in actual industrial scale unless such products could be further converted to a more value-added chemicals.

Last but not least, this work does not look at the purification of the acid-hydrolyzed products and therefore the reusability of the acid has not been investigated. This is proposed as a future work here as the success of such operation will give merits to the future application of this process.

Table 5.1 Economical analysis of *Ankistrodesmus* sp.

Hydrolysis before lipid extraction	Cost (THB)	Lipid extraction before hydrolysis	Cost (THB)
Hydrolysis 3 h		Lipid extraction 6 h	
Microalgae 0.5 g	0.3600	Microalgae 0.5 g	0.3600
Water 25 mL	0.0003	Solvent 10%vol. reused	0.1560
H_2SO_4 0.75%wt	0.0966	Electricity	0.1467
Electricity	0.6975		
Lipid extraction 4 h (hydrolyzed residue 0.175 g)		Hydrolysis 2 h (lipid extracted residue 0.424 g)	
Solvent 10%vol. reused	0.0702	Water 25 mL	0.0003
Electricity	0.0575	H_2SO_4 0.50%wt	0.0695
		Electricity	0.3341
Waste management		Waste management	
NaOH 0.009 g	0.0023	NaOH 0.006 g	0.0011
Total (THB/batch)	1.2844	**Total (THB/batch)**	1.0667

Products	Cost (THB)	**Products**	Cost (THB)
Glucose	0.0017	Glucose	0.0013
Xylose	0.0028	Xylose	0.0023
Acids	0.0007	Acids	0.0009
Protein	0.0560	Protein	0.0627
Lipid	0.0028	Lipid	0.0033
Total (THB/batch)	0.0639	**Total (THB/batch)**	0.0704

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APPENDIX

EXPERIMENTAL DATA FOR ANALYSIS

A-1 Standard calibration curve for HPLC analysis of glucose and xylose

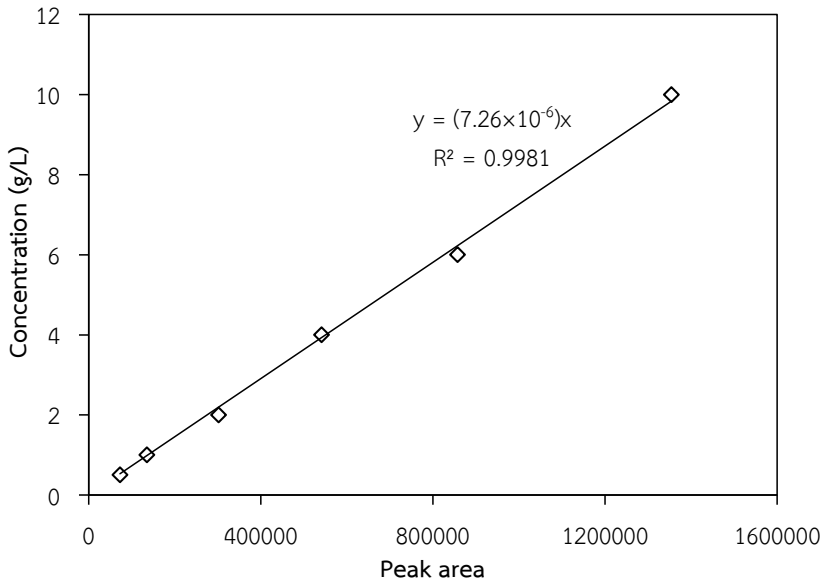

Figure A-1.1 Standard calibration curve of glucose

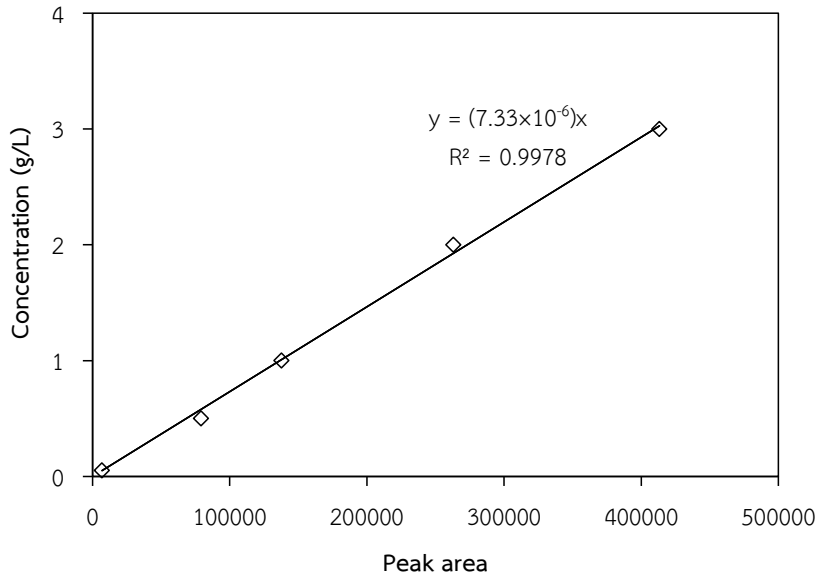

Figure A-1.2 Standard calibration curve of xylose

A-2 Standard calibration curve for HPLC analysis of sugar acids

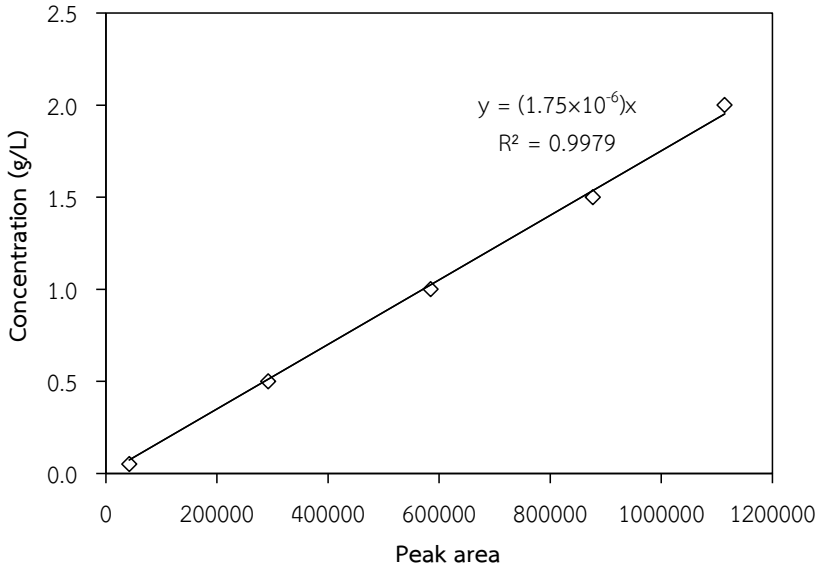

Figure A-2.1 Standard calibration curve of acetic acid

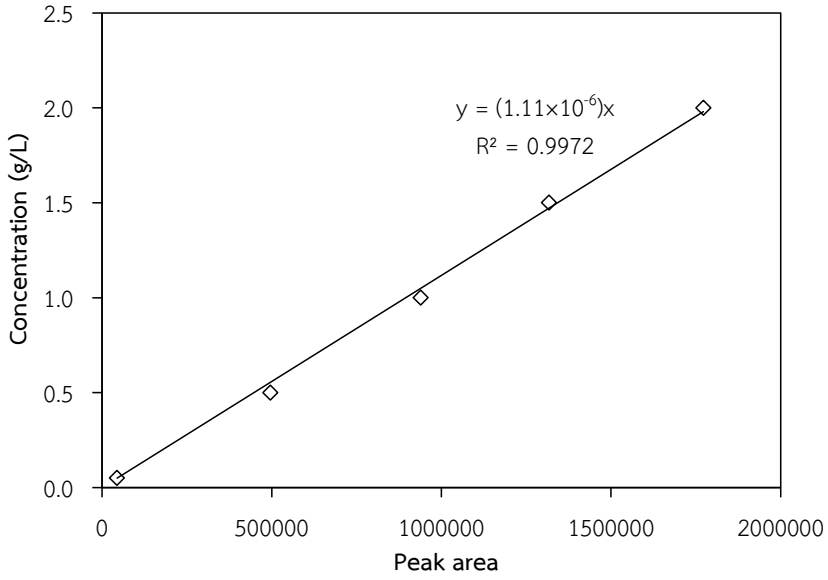

Figure A-2.2 Standard calibration curve of formic acid

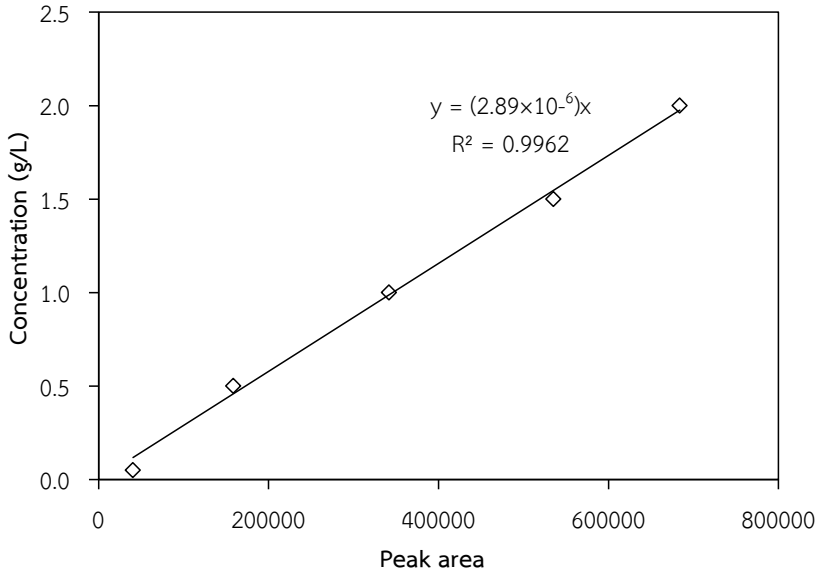

Figure A-2.3 Standard calibration curve of levulinic acid

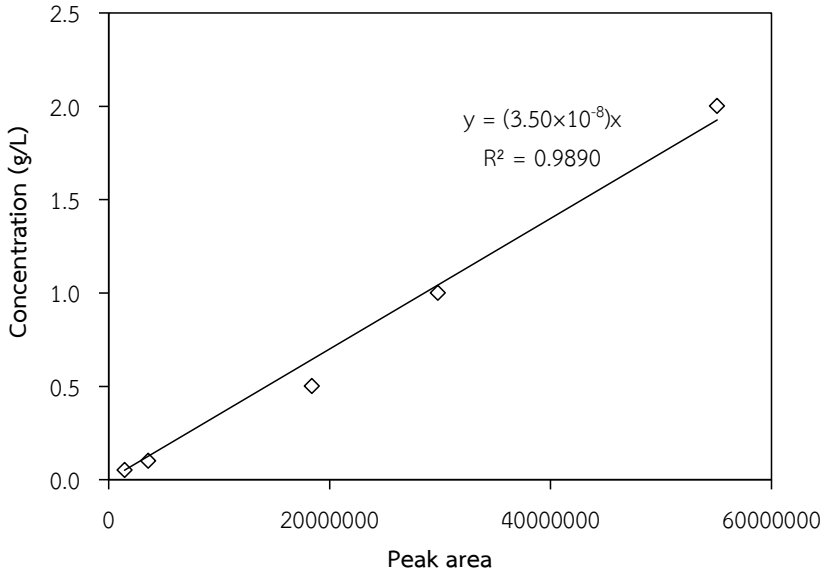

Figure A-2.4 Standard calibration curve of HMF

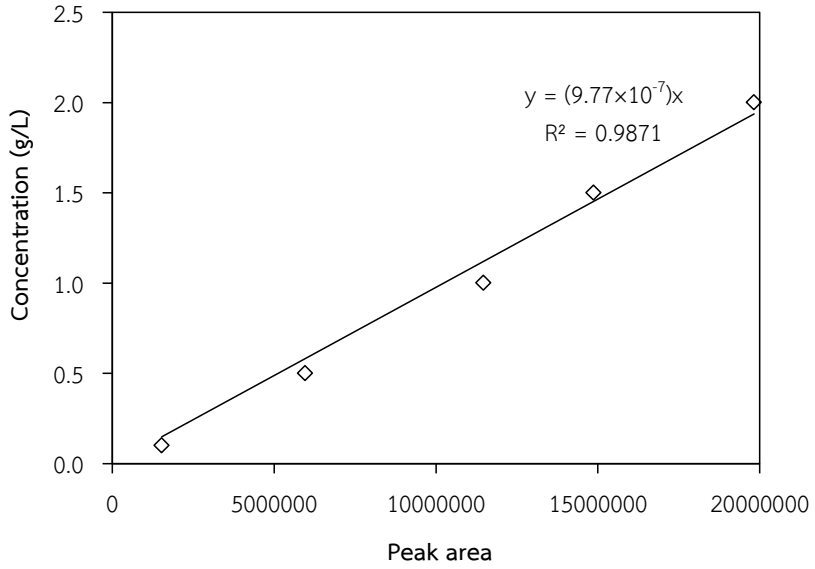

Figure A-2.5 Standard calibration curve of furfural

A-3 Standard calibration curve for protein analysis by using Lowry's method

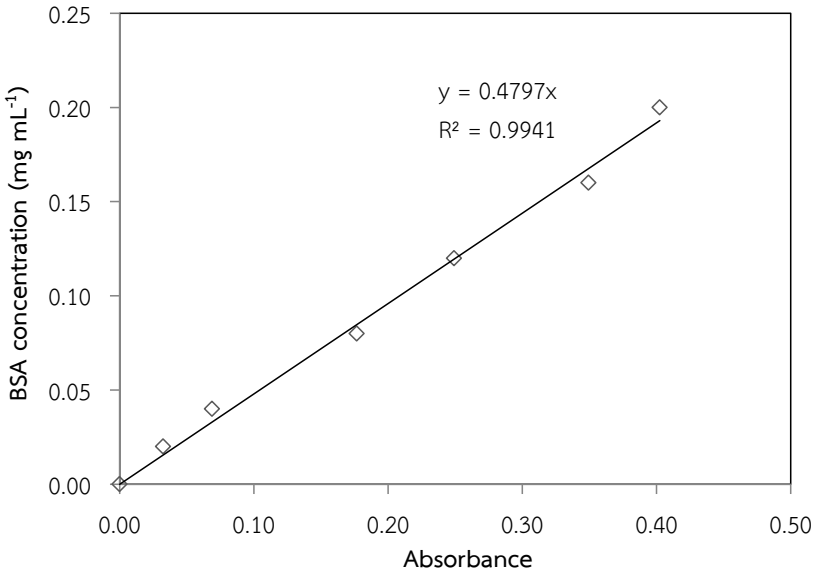

Figure A-3 Standard calibration curve of protein

VITA

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